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FINAL REPORT

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INTRODUCTION

Prostate cancer is the most common malignancy and the second leading cause of cancer death among men in the Western world. However, our understanding of the genetic events associated with the pathogenesis of this disease has lagged behind that for other common tumors. This knowledge deficit has contributed to the lack of development of new treatment strategies for prostate cancer. Several key oncogenes such as *Ras*, and tumor suppressor genes such as RB, p53, appear to be only infrequently mutated or deregulated in prostate cancer (1;2). Rather, prostate tumorigenesis appears to be promoted by the loss of as yet known genes (1;3;4). The high frequency of 8p12-21 loss in prostate carcinomas, combined with preliminary evidence of functional tumor suppression mediated by this chromosomal region, indicates that a gene (genes) located at or near 8p21 plays an essential role in the development of prostate cancer (3;4). The identification and characterization of these genes are thus essential for our understanding of the development of this important disease.

Several years ago, the PI's laboratory identified and characterized the human gene *PLK3* (alternatively named *PRK or FNK*) (5), which encodes a serine-threonine kinase (6). Plk3 protein shares sequence homology with *Sacchromyces cerevisiae CDC5* and *Drosophila melanoganster* Polo, both of which have been implicated in regulation of the G_2/M transition and M phase progression (7). Indeed, human Plk3 is capable of complementation of a temperature sensitive *CDC5* mutant of the budding yeast (6), suggesting that Plk3 also regulates M phase function. Northern blot analysis of specimens from 18 individuals with lung cancer revealed that *PLK3* mRNA is less abundant in tumor tissues than in normal tissues (5). Indeed, ectopic expression of *PLK3* reduces proliferation rate of transformed cells (8). Furthermore, in collaboration with a commercial partner, we observed several years ago that *PLK3* gene was localized to human chromosome 8p21 (8), a locus known to harbor prostate tumor susceptibility genes (3;4).

HYPOTHESIS AND OBJECTIVES

On the basis of the observations that the *PLK3* gene is down-regulated or mutated in certain prostate tumor cell lines, as well as other tumors (5;8;9), we hypothesize that *PLK3* is a tumor suppressor gene whose inactivation is an important event in the development of prostate cancer. We have proposed to characterize the genetic and epigenetic basis of aberrant *PLK3* transcripts detected in prostate cancer, their prevalence and possible clinical relevance; to test the

functional ability of *PLK3* as a tumor suppressor gene, and to investigate the physiologic function of *PLK3* by identification of proteins that interact with Plk3.

RESULTS

Task I. **To investigate the genetic and epigenetic basis of aberrant *PLK3* transcripts detected, their prevalence, and possible clinical relevance in prostate cancer**

A. Isolation of *PLK3* genomic clone. Through a combination of genomic library screening and PCR, we have isolated and characterized *PLK3* genomic locus. Fifteen exons throughout the open reading frame were characterized. None of the introns were exceptionally large, typically ranging from 100 to 300 base pairs in length. The sequences of exon 1 to intron 2 are highly GC rich. The results of these studies have been published (10).

B. Analysis of prostate cancer genomic DNA. As a first step to link *PLK3* to a tumor suppressor gene, we sought for functional inactivation of *PLK3* in various tumor cell lines. Total RNA was extracted. The first strand cDNA was synthesized, and used to amply the entire coding region of *PLK3* by PCR. The reaction products were cloned and sequenced. The summarized data is shown in Table 1. However, further analyses of genomic DNA from LACP9AD, PO7 and DU145 did not confirm the mutations (deletions) detected in *PLK3* transcript. Absence of *PLK3* was confirmed in Daudi, a lymphoblastic leukemic cell line (11).

Name	Histology	*PLK3*
LNCap	Prostate ca	Wild type
LACP9AD	Prostate ca	Deletion
PO7	Prostate ca	Del. Of 131 bp
WERI	Retinoblastoma	Del of 140 bp
DU145	Prostate ca	Nonsense159
R05	Renal cell ca	No product
Daudi	Lymphoblastic leuk.	No product

Table 1. Abnormal expression of *PLK3* in various tumor cell lines

C. Development of immunohistological analysis of the *PLK3* protein. We have developed several lines of monoclonal antibodies against Plk3. Their specificity was determined based on Western blot, immunoprecipitation, and immunofluorescence microscopic analyses as described (6;11-13). We have also developed a reliable method to study expression of Plk3 expression in tumor specimens via immunohistochemistry. Plk3 is primarily expressed in the cytoplasm of prostate epithelial cells (Fig. 1, brown staining). Poorly differentiated tissues contain no detectable levels of Plk3 antigen (Fig. 1B). As a control for Plk3 staining, a part of moderately differentiated tissues was also shown (Fig. 1B, lower right).

D. Alleotype analysis. During the course of our studies, we realized that our early report on the chromosomal localization of *PLK3* gene was not entirely correct (8). This is mainly due to the mistake by our contractor (SeeDNA Inc.) that did the FISH analysis. About a year ago, we confirmed, through BLAST search of databases of human genome, that *PLK3* gene is localized to 1p34 where loss of heterozygosity has been also detected in many types of cancer (14-16). However, this locus is not known for a hot spot of prostate cancer. Nonetheless, we have modified the objective by

studying regulation of *PLK3* expression in prostatic carcinoma cell lines given that *PLK3* expression is inducible by a variety of mitogens (5). LNCaP (androgen responsive) cells were treated with the synthetic androgen R1881 (1 nM) for various times, after which they were collected for isolation of total RNA. The abundance of *PLK3* mRNA was analyzed by Northern blotting. We observed that *PLK3* mRNA was inducible by androgen analog (Fig. 2), suggesting that *PLK3* gene may be involved in regulation of prostate (normal and pathological) development.

PLK3 may not function as a prostate tumor suppressor gene as originally proposed. However, available evidence indicates that *PLK3* is a general tumor susceptibility gene, down-regulation (or inactivation) of which predispose cells to malignant transformation. To further examine whether there existed a correlation between *PLK3* down regulation and tumor development, we examined *PLK3* expression in many types of primary tumors via immuno-histochemistry. Consistently, Plk3 levels were significantly lower in malignant tissues of stomach, rectum, liver and kidney (Fig. 3 & 4) as compared with those normal counterparts. Some levels of down-regulation of Plk3 also occurred in malignant lung, esophagus, colon, and breast (Fig. 3 & 4). These observations thus strongly suggest that *PLK3* may be a tumor suppressor gene suspected at the short arm of chromosome 1.

Task II. To test the functional ability of *PLK3* as a tumor suppressor gene

A. Transfection of *PLK3* into prostate cancer cells identified in Task I. We have obtained several *PLK3* expression constructs (wild-type as well as kinase-defective ones). These expression constructs were transiently transfected into GM00637 (fibroblast), A549 (lung carcinoma), LNCap (prostatic carcinoma), HT29 (colorectal carcinoma), HeLa (cervical carcinoma), and melanoma cell lines. Attempts were also made to transfect inducible *PLK3* expression constructs into LNCaP cells and the experiment was not successful.

B. Analysis of transfectants. We have reported that transfection of *PLK3* into fibroblast cells or lung carcinoma cells resulted in a decrease in cell proliferation and apoptosis (8). The effect of *PLK3* on induction of apoptosis was also confirmed in LNCaP (date not shown) and several other carcinoma cell lines as revealed by independent approaches such as DNA fragmentation, PARP (poly ADP-ribose polymerase) and micronuclei analyses (9;11;12) as well as morphological observations (data not shown). Our studies strongly suggest that *PLK3* plays an important role in negative regulation of cell proliferation.

C. To test the proteins translated from aberrant *PLK3* transcripts in assays for *PLK3* function. We have analyzed the effect of *PLK3* mutants ($Plk3^{K52R}$, $Plk3^{K52R-K57R}$, $Plk3^{K52R-K57R-K62R}$) on cell growth. We have observed that the kinase activity of Plk3 is necessary for apoptosis as well some other biological effects associated with Plk3 protein (11;12).

Genetically modified mice have significant potential for understanding the interactions between genotypic susceptibility. Recently, we have generated *PLK3* null mice through a gene trapping method (17). Mouse embryonic stem (ES) cells with a targeted disruption of the *PLK3* locus were obtained by a gene trapping method as

described (17). The targeting retroviral vector, which contained a neomycin resistance cassette, was inserted between exons 9 (E9) and 10 (E10) of *PLK3* (Fig. 5A). Two independent 129/Sv-derived ES cell lines were injected into BL/6 blastocysts, and the resulting chimeric mice were backcrossed to wild-type BL/6 animals. Disruption of *PLK3* in offspring was confirmed by Southern blot analysis with a probe targeted to *PLK3* (Fig. 5B). The genotypes of the offspring of various crosses were also determined by a polymerase chain reaction (PCR) strategy (Fig. 5A for primers' locations and orientations). A typical genotyping result was shown in Figure 5C. The genotyping analysis thus indicates that *PLK3* null pups are able to survive to term. These *PLK3* null mice will be examined for their susceptibility to spontaneous or carcinogen-induced tumor development.

D. Rescue CDC5 mutant yeast. We previously reported that human *PLK3* was capable of rescuing yeast CDC5 mutant (6), suggesting the functional conservation of *PLK3*. Because we developed many assays that are more convenient and effective than the yeast mutant rescue assay we have not used, as originally proposed, for the functional study of *PLK3*.

E. Germinal vesicle breakdown in *Xenopus* oocytes. Due to the same reason as described in D, we did not use this approach to analyze Plk3's function.

Task III. To investigate the physiologic function of PLK3 by the identification of proteins which interacts with Plk3

A-D. Study of proteins interacting with Plk3 using yeast two-hybrid system. We first generated plasmids that were essential for the study of proteins interacting with Plk3 via yeast two hybrid systems (detailed descriptions were as described (18;19). We have successfully identified several proteins that are implicated in regulating or mediating the function of Plk3 during cell cycle progression or DNA damage checkpoint control. Please refer to the attached paper for candidate proteins that are associated with Plk3 in yeast two-hybrid assay (18). Using other biochemical approaches (e.g., pull-down and coimmunoprecipitation) we have conformed that Chk2, a protein kinase important for DNA damage checkpoint response, physically interacts with Plk3 and the interaction is enhanced after DNA damage (11). Our further studies indicate that Plk3 may be downstream of Chk2 and that Plk3 is an important mediator in DNA damage response pathway (18).

E-. Other approaches. We have also identified proteins that are potentially in vivo targets of Plk3 and they are Cdc25C and p53 (11;20). Results of these studies have been published (see attached reprints). Our studies have defined a new component in the DNA damage and stress response pathways (11;13). We believe that the results of these studies will increase the understanding of the molecular basis of *PLK3* function during normal and malignant cell growth and that they will facilitate the identification of new molecular targets for cancer intervention.

Summary. We have made significant progress during the past several years in the understanding of the mode of action of *PLK3* in regulating cell proliferation. Although it turned

out that *PLK3* may not be a prostate cancer susceptibility gene as originally thought, *PLK3* does possess the characteristics of a general tumor suppressor. Therefore, the knowledge obtained should provide important insights into the molecular basis of the pathogenesis of many common malignancies.

Key Accomplishments

- *PLK3* mRNA expression is down-regulated in several types of common cancers.
- Plk3 protein levels were also lower in tumor tissues than their normal counterparts.
- Mutations in *PLK3* genes are infrequent events in cell lines derived from lung carcinomas although aberrant *PLK3* transcripts are detected in other types of tumor cell lines.
- Ectopic expression of *PLK3* results in apoptosis, which is likely due to its effect on microtubules.
- Plk3 interacts and phosphorylates Cdc25C and p53 in vitro, two important proteins involved in cell cycle regulation or cell cycle checkpoint control.
- Plk3 phosphorylates p53 on serine-20, a site important for activation of p53.

Reportable Outcomes

1. **Dai W**, Li Y, Ouyang B, Reissmann P, Li J, Wiest J, Stambrook P, Noffsinger A, Bejarano P. *PRK*, a cell cycle protein kinase gene, is localized to 8p21 and down-regulated in head/neck cancer. *Genes, Chromosomes & Cancer* 2000 27:332-336.
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7. Xie SQ, Wu HY, Wang W, Kunick J, Thomas RO, Hollingsworth RE, Cogswell J, **Dai W**. Genotoxic stress-induced activation of Plk3 is partly mediated by Chk2. *Cell Cycle* 2002 1:424-429

Conclusions

- Plk3 functions as a novel tumor suppressor gene.

- Plk3's tumor suppression function may be partly mediated through regulating the activity of p53.

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APPENDIX I

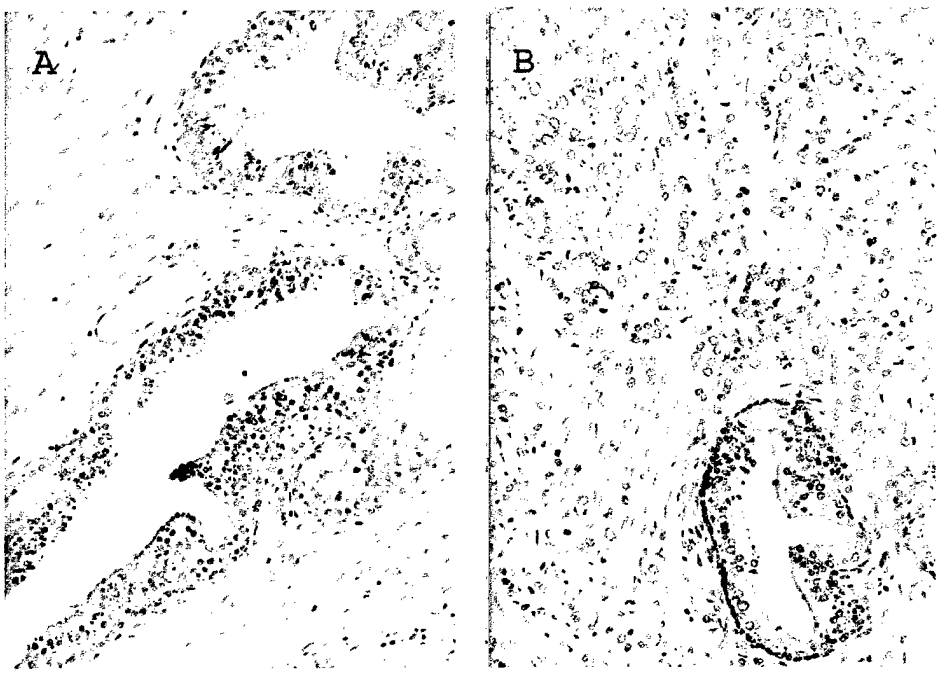
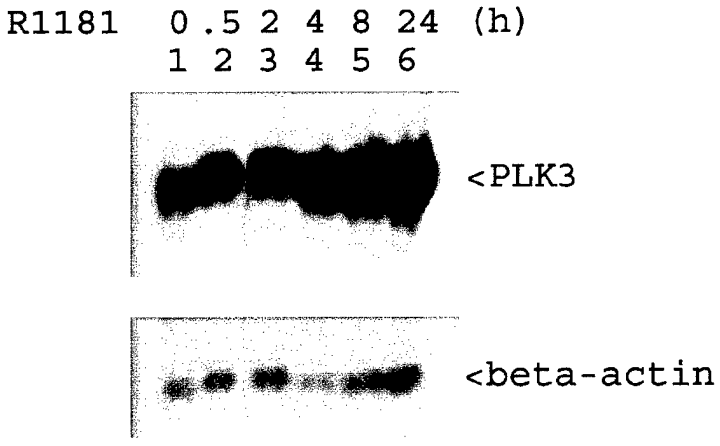


Figure 1



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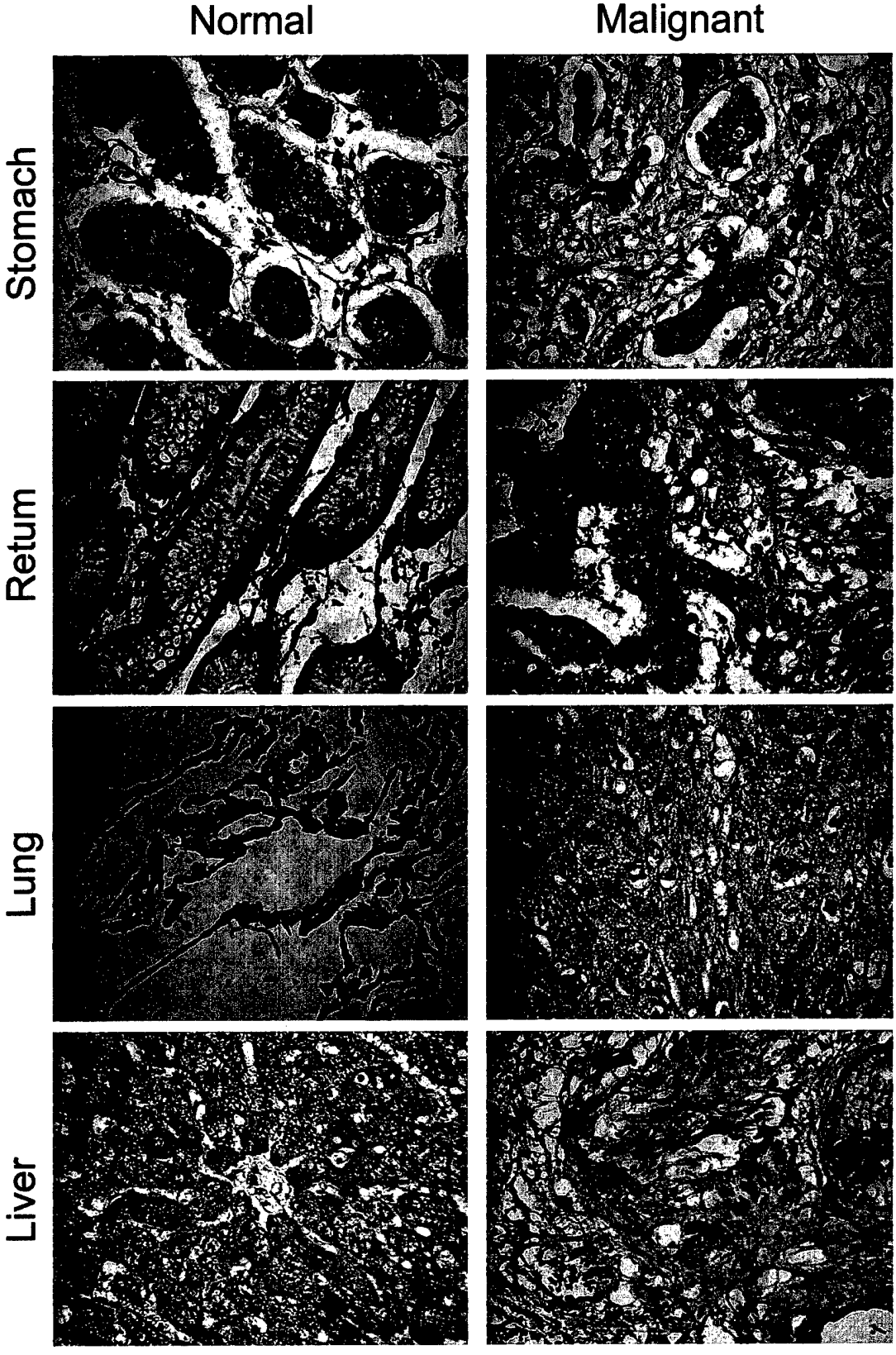

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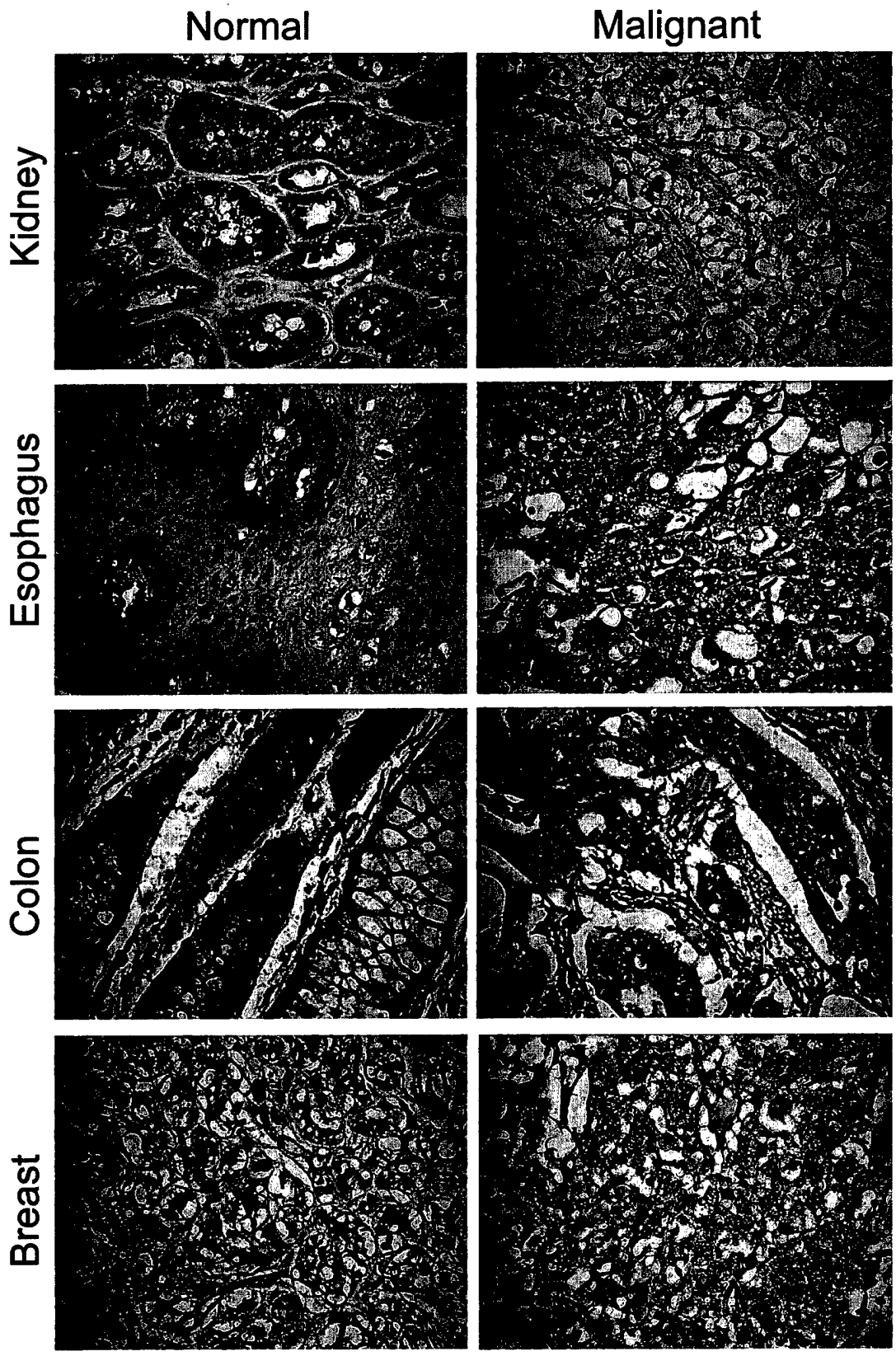

Figure 4

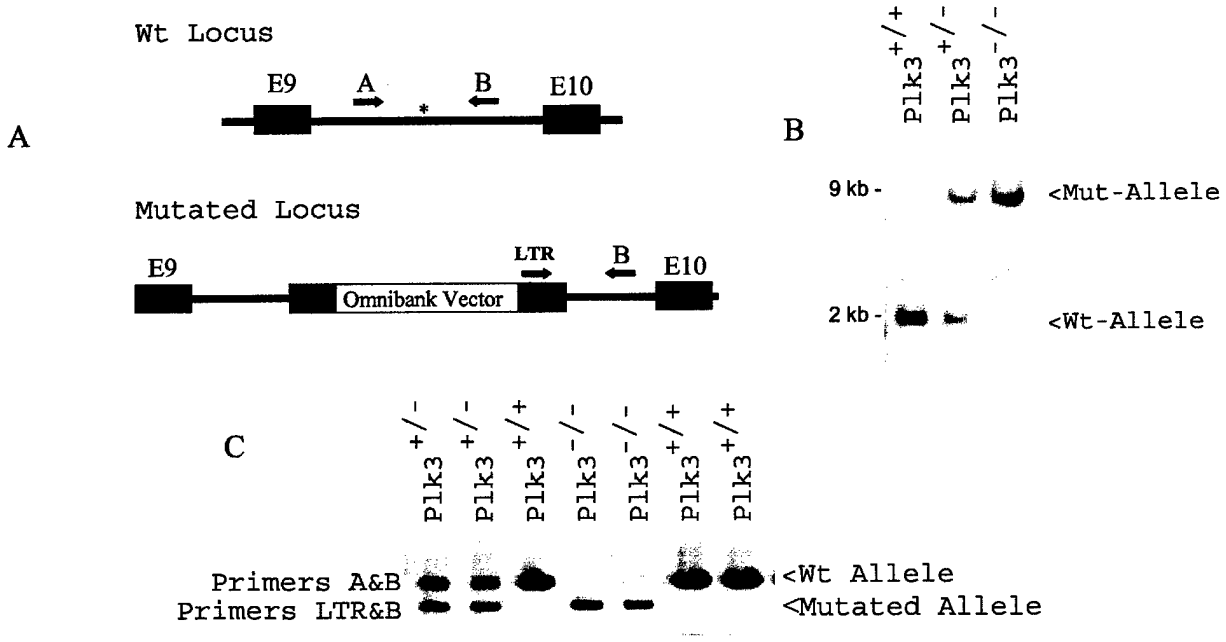

Figure 5. Disruption of mouse PLK3 locus. (A) Structures of the wild-type (Wt) and mutated PLK3 loci. The positions and relative orientations of three primers (A, B, and LTR) used for genotyping are shown. The star (*) denotes the insertion site. (B) Mouse genomic DNA was digested with Bgl II and then blotted with a BUBR1 DNA fragment labelled with ^{32}P. (B) Genotyping of mouse tail DNA by PCR. Genomic DNA isolated from the tails of offspring of inter-crosses between heterozygous ($PLK3^{+/-}$) mice was analyzed by PCR with primers A, B and LTR. The resulting products were analyzed on agarose gels.

APPENDIX II

cell cycle

Experimental Papers

Genotoxic Stress-Induced Activation of Plk3 is Partly Mediated by Chk2

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Plk3, p53, Genotoxic stress, Chk2, Protein Kinase

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Experimental Papers

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Raymond O. Thomas[2]
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ABSTRACT

Polo-like kinase 3 (Plk3, alternatively termed Prk) is involved in the regulation of DNA damage checkpoint as well as in M-phase function. Plk3 physically interacts with p53 and phosphorylates this tumor suppressor protein on serine-20, suggesting that the role of Plk3 in cell cycle progression is mediated, at least in part, through direct regulation of p53. Here we show that Plk3 is rapidly activated by reactive oxygen species in normal diploid fibroblast cells (WI-38), correlating with a subsequent increase in p53 protein level. Plk3 physically interacts with Chk2 and the interaction is enhanced upon DNA damage. In addition, Chk2 immunoprecipitated from cell lysates of Daudi (which expressed little Plk3) is capable of stimulating the kinase activity of purified recombinant Plk3 in vitro, and this stimulation is more pronounced when Plk3 is supplemented with Chk2 immunoprecipitated from Daudi after DNA damage. Furthermore, ectopic expression Chk2 activates cellular Plk3. Together, our studies suggest Chk2 may mediate direct activation of Plk3 in response to genotoxic stresses.

INTRODUCTION

Mammalian cells contain at least three proteins (Plk1, Plk2, and Plk3) that exhibit marked sequence homology to Polo.[1-4] As cells progress through the cell cycle, Plk proteins undergo substantial changes in abundance, kinase activity, or subcellular localization. In human cells, the amounts of Plk1 protein and of its kinase activity peak at mitosis.[2] During mitosis, Plk1 transiently associates with mitotic structures such as the spindle apparatus, kinetochores, and centrosomes.[5,6] Recent studies have shown that Plk1 contributes to a variety of mitotic (and meiotic) events, including phosphorylation of cyclin B and Cdc25C, activation of cyclin B-Cdc2 (CDK1), centrosome maturation, formation of the bipolar spindle at the onset of mitosis, and cytokinesis.[7,8] Plk1 also controls the exit of cells from mitosis by regulation of the anaphase-promoting complex and proteasomal activity.[9,10]

Plk3, originally cloned and characterized in our laboratory, shows distinct biological functions. The abundance of Plk3 remains relatively constant, as compared with that of Plk1, during the cell cycle and its kinase activity peaks during late S and G_2 phases.[11] Plk3 phosphorylates Cdc25C on serine-216,[12] phosphorylation of which is thought to inhibit the activity of this protein phosphatase whereas phosphorylation of Cdc25C by Plx1, a *Xenopus* Plk1 ortholog, results in activation of this protein.[13] In addition, the amount of Plk3 mRNA, but not that of Plk1 mRNA, is rapidly and transiently increased in response to mitogenic stimulation.[4]

In response to DNA damage, the kinase activity of Plk3 was rapidly increased in tumor, or transformed, cell lines in an ATM-dependent manner.[14,15] Peptide mapping as well as in vitro phosphorylation followed by immunoblot analysis with antibodies specific for phosphorylated forms of p53 indicated that Plk3 phosphorylates p53 on physiologically relevant sites.[14] Immunoprecipitation and "pull-down" assays show that Plk3 physically interacts with p53 and that the extent of this interaction is increased in response to DNA damage.[14] These results suggest that Plk3 functionally links DNA damage to the induction of cell cycle arrest or apoptosis.

The signaling pathways that underlie the cellular response to DNA damage (genotoxic stress) consist of sensors, signal transducers, and effectors.[16] Although the identities of the damage sensors remain unknown, the molecular entities responsible for transducing the damage signals to specific effectors are relatively well characterized. ATM (mutated in ataxia telangiectasia) and its homolog ATR (ATM-related) function early in the signaling pathways and are central to the DNA damage response.[16] Downstream targets (substrates)

of ATM and ATR include the protein kinases Chk1 and Chk2,[17-19] effector molecules that execute the DNA damage response include p53 and Cdc25C. ATM and ATR, together with Chk2 and Chk1, respectively, are thought to mediate the specific cellular responses to different types of DNA damage in mammalian cells.

Polo family kinases also participate in the response to DNA damage.[18,20] Cdc5 promotes adaptation to cell cycle arrest at the DNA damage checkpoint in *Saccharomyces cerevisiae*.[21] The electrophoretic mobility of Cdc5 in denaturing gels is affected by prior subjection of cells to DNA damage, and this modification is dependent on Mec1, Rad53 (a yeast Chk1 homolog), and Rad9.[20] In addition, a functionally defective Cdc5 mutant protein suppresses a Rad53 checkpoint defect, whereas overexpression of Cdc5 overrides checkpoint-induced cell cycle arrest,[18] suggesting that Cdc5 acts downstream of Rad53. Moreover, DNA damage appears to interfere with the activation of Plk1 in mammalian cells, resulting in down-regulation of the kinase activity of this protein.[18] Here we report that Plk3 is activated in normal diploid fibroblast cells after exposure to reactive oxygen species and that genotoxic stress-induced Plk3 activation appears to be partly mediated by Chk2.

MATERIALS AND METHODS

Cell Lines. WI-38 (normal diploid fibroblast), A549 (lung carcinoma) and Daudi (lymphoblastic leukemia) cells were purchased from ATCC. GM00637 cell line (transformed human fibroblast) was originally obtained from the Coriell Institute for Medical Research. Cells were cultured in culture dishes in appropriate media supplemented with 10% fetal bovine serum and antibiotics (100 µg/ml penicillin and 50 µg/ml streptomycin sulfate) with 5% CO_2.

Yeast Two-Hybrid Assays. Yeast two-hybrid analysis was performed as described previously.[22] The *PLK3* gene was subcloned into the EcoR1-XhoI sites of the two-hybrid bait vector pMW101, and this bait was used to screen prey libraries derived from fetal brain, fetal liver, testis, macrophage, and prostate tissues. Expression of the bait was verified by western blotting. Interactions were confirmed and determined to be specific by repeat yeast two-hybrid analysis testing baits against preys directly.

Pull-Down Assays and Immunoblotting. WI-38 cells treated with H_20_2 (200 µM) for various times were collected and lysed in a lysis buffer as described.[11] Equal amounts of protein lysates were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies to p53 as described.[14] For pull-down assays, recombinant His6-Plk3 expressed with the use of baculoviral expression system as described[12] was affinity-purified with and subsequently conjugated to Ni^{2+}-NTA resin. Plk3-conjugagted Ni^{2+}-NTA resin or the resin alone was incubated for 3 hours with lysates (1 mg of protein) of A549 cells that had been infected with null adenovirus or Chk2 adenovirus for 16 hours and treated with or without adriamycin for 1 hour. After washing of the resin, bound proteins were eluted and subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis with antibodies to Chk2 or p53. The signals were detected with horse-radish peroxidase-conjugated goat secondary antibodies and enhanced chemiluminescence reagents.

Immunoprecipitation. A549 or GM00637 cell lysates (~2 mg of protein) were incubated for 30 min at room temperature in a total volume of 500 µl with of 20 µl of protein A/G-agarose bead slurry (Santa Cruz Biotech). After removal of the beads, the supernatant was supplemented with either with Plk3 immunoglobulin (IgG) or a control IgG (against Tyro-3), followed by incubation for an additional 2 h at room temperature. Protein A/G–agarose beads (20 µl) were then added to each immunoprecipitation mixture and the incubation was continued for 1 h at room temperature. Immunoprecipitates were collected by centrifugation, washed three times with the cell lysis buffer, and subjected to immunoblot analysis with the antibody to Chk2.

Immunocomplex Kinase Assays. Immunocomplex kinase assays were performed essentially as described.[14] In brief, cells were treated with H202 (200 µM) for various times, lysed, and subjected to immunoprecipitation with antibodies to Plk3. The resulting precipitates were resuspended in a kinase buffer [10 mM Hepes-NaOH (pH 7.4), 10 µM MnCl2, 5 mM $MgCl_2$; 25 µM ATP], and the kinase reaction was initiated by the addition of [γ-^{32}P]ATP (2 µCi) (Amersham) and α-casein (Sigma). After incubation for 30 min at 37°C, the reaction mixtures were analyzed by SDS-PAGE and autoradiography. Recombinant His_6-Plk3 was also assayed for kinase activity as controls. Each assay was repeated at least once.

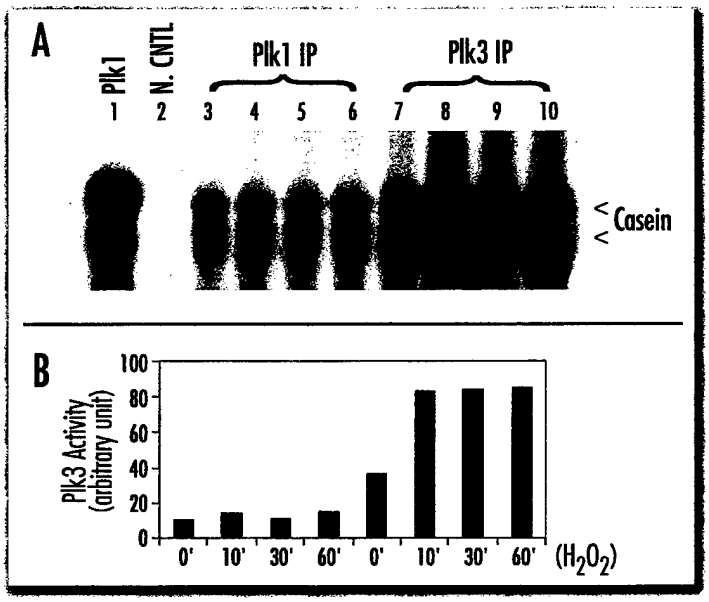

Figure 1. Activation of Plk3 in normal diploid fibroblast cells by H2O2. (A) WI-38 cells were treated with H2O2 for 0, 10, 30 or 60 minutes. An equal amount of cell lysates (0.5 mg) from various treatments was subjected to immunoprecipitation using antibodies against Plk3 or Plk1. Plk3 or Plk1 immunoprecipitates were analyzed for kinase activities using casein as substrate. Recombinant Plk1 (lane 1) was used as a positive control and the kinase reaction supplemented with casein but no Plk1 (lane 2) was used as a negative control for the kinase assay. (B) Quantification of Plk1 and Plk3 kinase activities as shown in A.

RESULTS

We have previously shown that GM00637 and A549 cells exposed to genotoxic stresses contained activated Plk3.[15] We have also obtained evidence indicating that Plk3 may directly regulate p53 by phosphorylation upon activation of DNA damage checkpoint in these cell lines.[14] However, GM00637 cells have acquired an unlimited life span (immortalized) by SV40 virus and A549 cells were derived from a human lung carcinoma. It is known that p53 is deregulated in the presence of SV40 viral antigen or due to oncogenic transformation.[23] Therefore, we further investigated whether the activation of Plk3 also occurs in normal diploid cells. WI-38 (normal diploid fibroblast) cells treated with H_20_2 for various times were analyzed for the kinase activity of Plk3, as well as Plk1. We observed that there exited in the cells a basal level of Plk3 activity, which was rapidly activated after H_20_2 treatment, reaching plateau about ten minutes of treatment. In contrast, Plk1, sharing significant structural homology with Plk3,[4] was not activated by H_20_2. We then analyzed p53 activation by measuring its protein level in cells treated with H_20_2 for various times. WI-38 cells contained a low level of p53. After H_20_2 treatment, the p53 level steadily increased. By 4 hours of treatment, p53 protein levels increased by approximately 8 folds. The observations that Plk3 activation followed by an increase in p53 levels in WI-38 cells treated with H_20_2 are consistent with our model as described previously,[14] proposing that p53 is downstream of Plk3 and that it may be a direct in vivo target of Plk3 during DNA damage response.

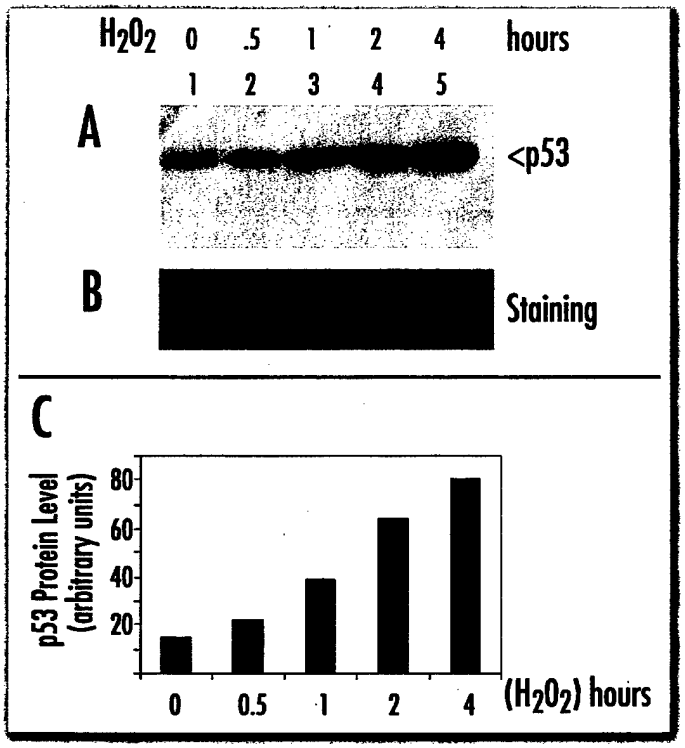

Figure 2. Activation of p53 in normal diploid fibroblast cells by H2O2. (A) WI-38 cells were treated with H2O2 for 0.5, 1, 2, or 4 hours. Cell lysates were prepared after treatment and equal amounts of cell lysates were blotted for p53 expression. (B) The same blot as shown A was stained as a loading control. (C) Quantification of p53 levels as shown in A.

To identify potential upstream regulators and/or new substrates of Plk3, we then employed yeast two-hybrid assay system to screen candidate proteins that physically interacted with this kinase. Among the approximately 30 interactors recovered upon yeast two-hybrid screening with Plk3, one was identical to the C-terminal half of Plk1 (amino acids 363–603), two contained overlapping portions of Chk2, one was a portion of ribonucleotide reductase M2, two comprised the catalytic subunit of protein phosphatase 1α, and one comprised a portion of Tensin2 (Table 1). In addition, we found that a protein of unknown function, KIAA1784 (GenPep Q96JP1), interacted with Plk3. The observation that Chk2 was among the candidate proteins interacting with Plk3 prompted us to study whether these two proteins were physically associated with each other in mammalian cells. Coimmunoprecipitation shows (Fig. 3A) that whereas control IgGs failed to bring down any Chk2 antigen Plk3 antibody precipitated Chk2 protein from both GM00637 and A549 cell lysates. Furthermore, pull-down assays revealed (Fig. 3B) that Plk3 resin, but not the control

Table 1 **Proteins That Interact with Plk3 in Yeast Two-Hybrid Screening Assays**

	# of Clones	Name
Known function		
	1	Plk1
	2	Chk2
	1	Ribonucleotide reductase R2
	2	Protein phosphatase 1a
	1	Tensin2
Unknown function		
	1	KIAA1784 (GenPep Q96JP1)

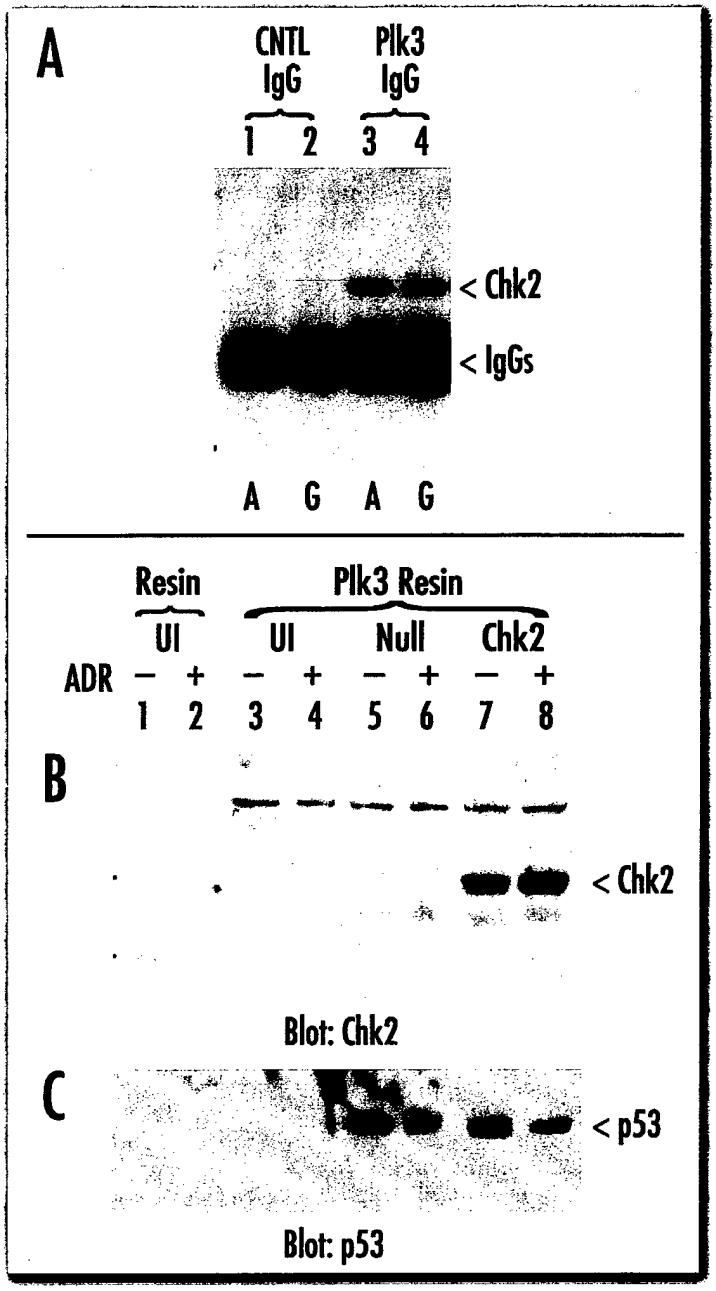

Figure 3. Chk2 interacts with Plk3. (A) A549 ,'A',or GM00637, 'G', cell lysates were immunoprecipitated with the antibody to Plk3 or to Tyro-3 as a negative control (CNTL). The immunoprecipitates were blotted for Chk2. Arrows indicate the positions of Chk2 and immunoglobulins (IgGs). (B) A549 cells were infected with recombinant adenovirus expressing Chk2 or with null adenovirus. One day after infection, the cell lysates were prepared and an equal amount of the protein lysates, along with the lysates from noninfected cells, were incubated with Ni^{2+}-NTA resin conjugated with Plk3. Ni^{2+}-NTA resin incubated with uninfected, 'UI', cell lysates were used as a control. After washing, proteins interacting with Plk3 or control resin were eluted and subjected to immunoblotting analysis for Chk2. (C)The same blot as shown in (B) was stripped and reprobed with an antibody to p53.

resin, was capable of precipitating ectopically expressed Chk2, indicating a true interaction between Plk3 and Chk2. Moreover, this interaction was enhanced after treatment with adriamycin (Fig. 3B), a radiomimetic drug causing DNA strand break. Plk3 was unable to pull-down significant amounts of endogenous Chk2 because it is low in these cells and because a small amount (250 μg) of cell lysates (2 mg for Co-IP) was used for the

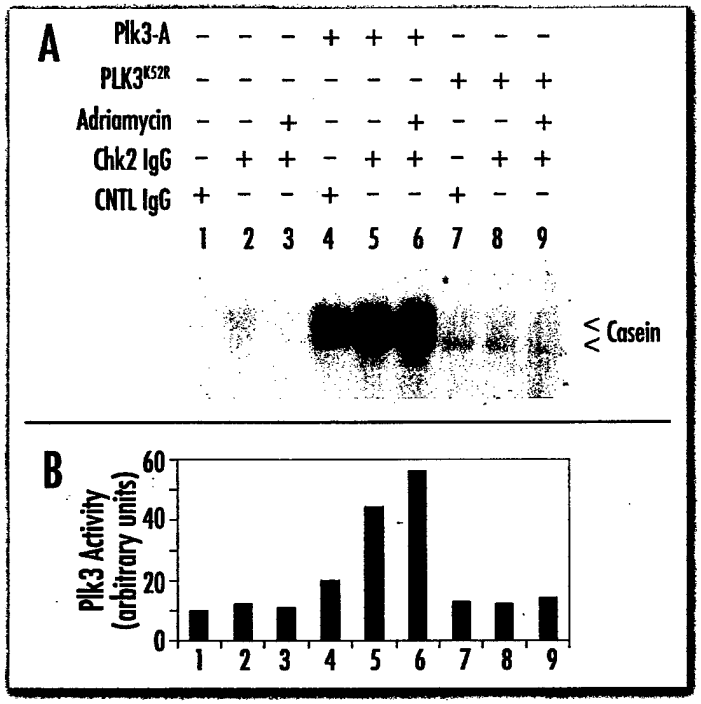

Figure 4. Chk2 activates Plk3 kinase activity in vitro. (A) Chk2 immunoprecipitated from Daudi cells treated with or without adriamycin was analyzed for kinase activities toward α-casein. Additional Chk2 immunoprecipitates from an equal amount of Daudi cell lysates were added to the kinase reaction mixture containing Plk3-A or Plk3^{K52R}. After kinase reaction, the mixtures were fractionated on SDS-PAGE followed by autoradiography. (B) Quantification of the kinase activities as shown in A.

assay. Given that both Plk3 and Chk2 are reported to interact with and phosphorylate p53,[14,16] we examined whether the observed interaction between Chk2 and Plk3 is direct or via p53. The same blot as shown in Fig. 3B was stripped and blotted for p53. It was shown (Fig. 3C) that Plk3 resin did not precipitate detectable amounts of p53 from non-infected A549 cells. On the other hand, Plk3 brought down a significant amount of p53 in cells infected with either null or Chk2 adenovirus, indicating that viral infection alone induced an interaction between Plk3 and p53. However, Plk3 showed no enhanced interaction with p53 after adriamycin treatment. These combined observations suggest that the interaction between Plk3 and Chk2 is largely p53-independent.

To investigate whether Chk2 directly regulates Plk3 kinase activity we supplemented Plk3 kinase reaction mixtures with Chk2 immunoprecipitated from Daudi cells treated with or without adriamycin. Daudi cells were selected because these cells do not contain detectable levels of Plk3 protein.[14] Figure 4 shows that kinase-active Plk3 (Plk3-A), but not kinase-defective one (Plk3^{K52R}), significantly phosphorylated α-casein in vitro. Whereas Chk2 alone did not phosphorylate α-casein, it stimulated Plk3 kinase activity in vitro. The stimulation was more pronounced after addition of Chk2 immunoprecipitated from adriamycin-treated Daudi cells. In addition, Chk2 immunoprecipitated from either untreated or adriamycin-treated cells did not stimulate the kinase activity of Plk3^{K52R}.

To further determine whether Chk2 regulated Plk3 kinase activity in vivo we analyzed the activity of Plk3 directly immunoprecipitated from A549 cells infected with either Chk2 adenovirus or null adenovirus. Compared with that of non-infected cells, null adenovirus infection did not cause a significant change in Plk3 kinase activity (Fig. 5). However, Plk3 activity was greatly stimulated after infection with Chk2 adenovirus, supporting the notion that Chk2 may be upstream of Plk3 by activating Plk3 in vivo during response to certain genotoxic stresses.

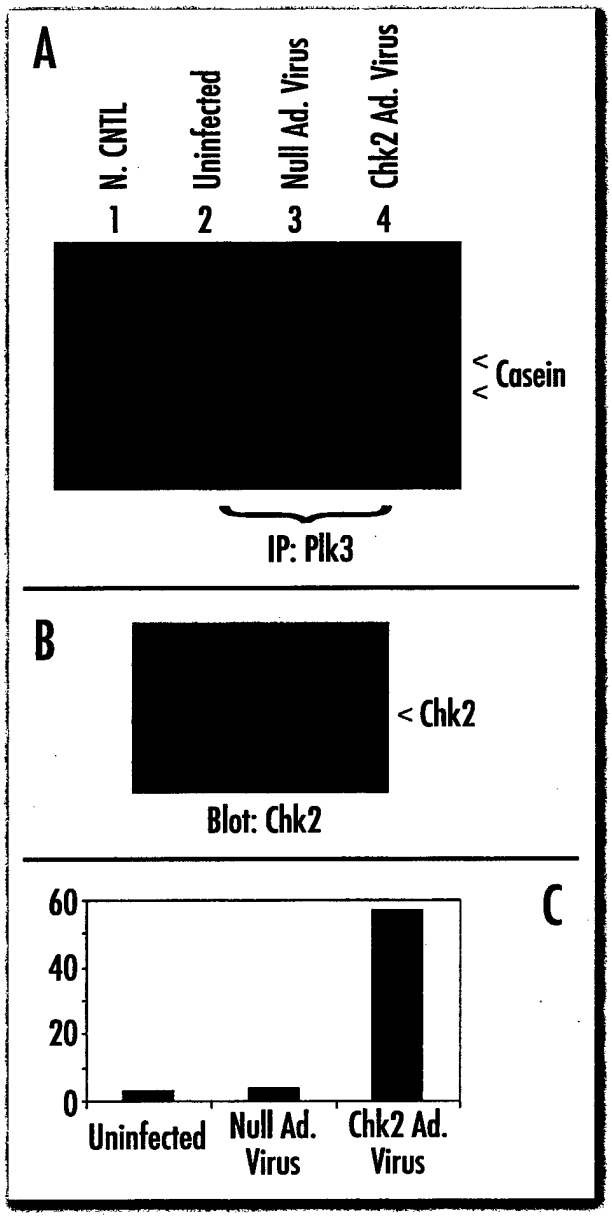

Figure 5. Chk2 activates Plk3 in vivo. (A) A549 cells were infected with either null or Chk2 adenovirus. One day after infection, cell lysates were immunoprecipitated with the antibody to Plk3. Plk3 immunoprecipitates were directly assayed for kinase activities toward α-casein. (B) Western blot analysis for Chk2 expression in uninfected cells or cells infected with null or Chk2 adenovirus. (C) Quantification of kinase activity as shown in A.

DISCUSSION

Plk3 and p53 are rapidly activated in normal diploid fibroblast cells after H2O2 treatment. This is consistent with our previous report that DNA damage caused by adriamycin or UV activates Plk3 as well as p53 in tumor cells or transformed fibroblast cells.[14] Thus, our studies establish that Plk3 is an important physiological player in response to a variety of genotoxic stresses. We have previously proposed that Plk3, a component in the DNA damage checkpoint response pathway, regulates both cell cycle arrest and apoptosis.[14] In the presence of DNA damage signals, cell cycle progression is halted to allow the cell time for repair. If the extent of DNA damage is beyond repair, cells commit suicide by activation of apoptosis, a

process that is likely mediated by Plk3 as well as p53. The existing evidence suggests that Plk3 may regulate cell cycle arrest and apoptosis through direct targeting Cdc25C and p53, respectively.[12,14]

Given that Plk3 activation is ATM-dependent,[15] Chk2, also downstream of ATM, may control Plk3 kinase activity or vice versa during DNA damage checkpoint activation. The observations that both Chk2 and Plk3 phosphorylate p53 on serine-20 in vitro lead us to propose that Plk3 may reinforce the DNA damage checkpoint,[14] which is otherwise primarily regulated by Chk2 and Chk1.[16] Because Plk3 strongly interacts with serine-20-phosphorylated p53,[14] we have also proposed that p53 phosphorylated on serine-20 by Chk2 would interact to Plk3 with high affinity, resulting in phosphorylation of p53 on additional sites. This is supported by our observation that Plk3 phosphorylates p53 on at least two major sites.[14] The latter scenario would place Plk3 downstream of Chk2 in the DNA damage checkpoint pathway. Our current studies support the possible regulatory hierarchy of Chk2 and Plk3.

1. Chk2 protein stimulates Plk3 kinase activity in vitro and the stimulation of Plk3 is more pronounced when activated Chk2 is supplemented to the kinase reaction.
2. Ectopic expression of Chk2 results in activation of Plk3. However, at this moment one could not eliminate the possibility that Plk3 may phosphorylate Chk2 and thus regulate its activity upon DNA damage.

We have previously shown that Plk3 interacts with p53 and the interaction is enhanced after DNA damage in GM00637 cells.[14] Different from GM00637 cells, A549 cells contain a low level of p53 (data not shown), which may explain why we were unable to detect a constitutive interaction between Plk3 and p53 (Fig. 3C). However, upon adenovirus infection the interaction between Plk3 and p53 is apparent, which is mostly likely due to stabilization of p53 and thus elevated p53 levels in virus-infected cells. Interestingly, adriamycin treatment did not enhance the interaction between Plk3 and p53 in adenovirus-infected cells. The affinity between Plk3 and Chk2 is relatively low because Plk3 only pulls down a significant amount of Chk2 in cells with ectopically expressed Chk2 (Fig. 3B). The fact that adriamycin is capable of stimulating the interaction between Plk3 and Chk2 but not between Plk3 and p53 indicates that the interaction between Plk3 and Chk2 is independent of p53.

We have recently demonstrated that Plk3 is localized to the centrosomal region of the cell and that it may participate in the regulation of microtubule dynamics as well as duplication of centrosome, the major microtubule organization center.[24] Proteins that participate in DNA repair or the DNA damage checkpoint also contribute to regulation of microtubule or centrosome function. For example, p53 exhibits centrosomal localization and deregulated expression of p53 or its mutations contributes to centrosome amplification,[25-27] the common subcellualr localization of which may account for our observed physical interaction between Plk3 and p53. In addition, ribonucleotide reductase R2 (small subunit) is involved in the DNA damage checkpoint response.[28] It forms active ribonucleotide reductase in vivo with R1 (large subunit), the latter of which is also an activator of microtubule nucleation.[29] Interestingly, yeast two-hybrid assays show that the small subunit of ribonucleotide reductase interacts with Plk3 (Table 1). It is tempting to speculate that centrosome duplication needs to be tightly coordinated with genome replication. DNA damage-induced cell cycle arrest or apoptosis should also result in a halt in centrosome duplication and maturation. Therefore, Plk3 may be an important component that controls centrosomal function in response to various genotoxic stresses.

A loss of checkpoint function frequently results in infidelity of inheritance of genetic information, and thereby predisposes cells to genetic instability and neoplastic transformation. Consistent with this, deregulated expression of Plk3 is reported in carcinomas of lung and head/heck.[4,30] In addition, Plk3 expression is significantly down-regulated in carcinogen-induced rat intestinal tumors.[31] Furthermore, the human Plk3 gene (PLK3) is localized to the short arm of chromosome 1 (1p34), a region that displays loss of heterozygosity or homozygous deletions in many types of cancers and which has been proposed to harbor tumor susceptibility genes.[32-34]

Acknowledgments

We thank members of the Dai lab for various suggestions.

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GENES, CHROMOSOMES & CANCER 32:384–389 (2001)

BRIEF COMMUNICATION

Intron/Exon Organization and Polymorphisms of the *PLK3/PRK* Gene in Human Lung Carcinoma Cell Lines

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PLK3/PRK, a conserved polo family protein serine/threonine kinase, plays a significant role at the onset of mitosis and mitotic progression. Recently, *PLK3/PRK* has been shown to induce apoptosis when overexpressed in cell lines and is also implicated in cell proliferation and tumor development. Forty lung tumor cell lines were used for single-strand confirmation polymorphism (SSCP) analysis and DNA sequencing to examine the mutational status of *PLK3/PRK*. No missense or nonsense mutations were revealed in the lung carcinoma cell lines examined. However, three polymorphisms were identified as: a G to A at position 720, an A to G at 1053, and a G to C at 1275. Intron/exon boundaries were determined by amplification of genomic DNA with *PLK3/PRK* exon-specific primers. The amplification products with increased size relative to the cDNA were sequenced. Fifteen exons throughout the open reading frame were characterized. None of the introns were exceptionally large, typically ranging from 100–300 basepairs in length. These results suggest that although *PLK3/PRK* expression is downregulated in a majority of lung carcinoma samples, mutational inactivation of the coding sequence of the *PLK3/PRK* gene appears to be a rare event in lung cancer.

Lung cancer is the leading cause of cancer-related mortality in the United States and Western Europe. It is postulated that 10–20 genetic alterations are required in tumorigenesis (Fearon and Vogelstein, 1990). Chromosome arm 8p is a frequent site of allelic loss in a wide range of epithelial cancers, including breast (Kerangueven et al., 1997), colorectal (Halling et al., 1999), head and neck (Li et al., 1994), prostate (Vocke et al., 1996), and lung cancer (Emi et al., 1992; Ohata et al., 1993; Lerebours et al., 1999; Wistuba et al., 1999; Girard et al., 2000). In fact, loss of heterozygosity on chromosome arm 8p was found to be an early and frequent event in lung tumorigenesis (Wistuba et al., 1999). The authors also reported that the size of the deletion increased with the severity of the histopathological preneoplastic changes (Wistuba et al., 1999).

A gene previously designated *PLK3/PRK* (for polo-like kinase / polo-related kinase) was localized to the chromosomal region of 8p21 by fluorescence in situ hybridization (Dai et al., 2000). *PLK3/PRK* is a member of an emerging family of protein kinases (designated the polo kinase family) described in yeast, *Drosophila, Xenopus*, mouse, and humans (Nigg, 1998). The polo family kinases are important in regulating the onset of mitosis and M-phase progression (Nigg, 1998). Mutants of the polo gene in *Drosophila* induce hypercondensed chromosomes and abnormal spindle formation (Fenton and Glover, 1993). A polo homolog encoded by *CDC5* in budding yeast is required for nuclear division in late mitosis (Kitada et al., 1993) and for adaptation to DNA damage response (Toczyski et al., 1997). Recently, it has been shown that a polo-like kinase (*PLX*) from *Xenopus* interacts with, phosphorylates, and activates the *CDC25* gene product (Kumagai and Dunphy, 1996), a dual specific protein phosphatase that, in turn, activates $p34^{cdc2}$. Mammalian PLK1 (polo-like kinase 1) plays an important role in several stages of mitosis. Besides contribution to the activation of cyclin B/CDC2 and the maturation of centrosomes (Nigg, 1998), PLK1 also controls mitotic progression by regulating anaphase-promoting complex (Kotani et al., 1998) and is involved in DNA-damage response (Smits et al., 2000). The human *PLK3/PRK* gene, displaying strong homology to polo family kinases, was downregulated in lung and head and neck carcinomas (Li et al., 1996). The PLK3/PRK protein shares a considerable sequence identity with

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TABLE I. Primer Pairs and Locations in *Prk*

SSCP-PCR primers		
Sense primer	Anti-sense primer	Region amplified*
5′ GCTGGCCGGGCTACCGAC 3′	5′ AGATTGGGTCAGCGTCCT 3′	39 to 325
5′ GCCTACGCTGTCAAATC 3′	5′ GTTCCAGTGTCTCAGTGATG 3′	178 to 514
5′ CAGCGCGGCATCTTGCAC 3′	5′ CAGCGTGTAGTGAACCT 3′	451–756
5′ GAGACGTACCGCATC 3′	5′ GGCCAACGGATGTGCGCA 3′	718 to 1063
5′ CCAGGAGAGGGATGAGGTC 3′	5′ ACGGCGGCTGGACAGTTG 3′	1006 to 1371
5′ CCAATAAGTTCGGCTTTG 3′	5′ CTGGACAGTGCCATCACT 3′	1331 to 1668
5′ GTCAAGACGGATCAGGCT 3′	5′ GAGCCTGACAGGCACAGG 3′	1618 to 1902

Long Distance PCR Primers	
Sense primer	Anti-sense primer
5′ ACTGACACAGAGACTGGCAGC 3′	5′ CAAATCCCCCACCTTCAGTTCC 3′
5′ CTTTGGAGACGGCTGACCTGA 3′	5′ AGACCTCATCCCTATCCTGG 3′
5′ GCTCTGAGAAATTGTATAGC 3′	5′ TCCATGTAGGAGGCGAAGT 3′

*Numbers given refer to the cDNA sequence for *Prk* in Li et al. (1996).

the *CDC5* gene product and is capable of complementing yeast *CDC5* temperature-sensitive mutants (Ouyang et al., 1997), suggesting that it is a functional homolog of the budding yeast *CDC5*. Our studies indicate that *PLK3/PRK* has an M-phase function (Ouyang et al., 1997). We have recently shown that PLK3/PRK interacts with and phosphorylates CDC25C (Ouyang et al., 1999). The phosphorylation of CDC25C by PLK3/PRK is at serine^{216}, a site also phosphorylated by Chk1 and Chk2, suggesting that PLK3/PRK may also be involved in DNA-damage response.

We have previously shown that *PLK3/PRK* mRNA levels were downregulated in lung and head/neck carcinomas (Dai et al., 2000). In addition, expression following transfection of the *PLK3/PRK* gene results in a decreased rate of proliferation for cells in culture (Dai et al., 2000; Conn et al., 2000). Considering that the *PLK3/PRK* gene is localized to chromosome band 8p21 (Dai et al., 2000), a locus suspected of harboring a tumor suppressor gene involved in numerous tumor types (Emi et al., 1992; Ohata et al., 1993; Li et al., 1994; Vocke et al., 1996; Kerangueven et al., 1997; Lerebours et al., 1999; Wistuba et al., 1999), we examined the mutational status of *PLK3/PRK* in cell lines derived from lung cancers. Total RNA was isolated from 40 lung cancer cell lines to test for polymorphisms or inactivating mutations. The following cell lines were used in these experiments: H28, H69, H125, H157, H187, H324, H345, H372, H378, H446, H460, H526, H661, H719, H720, H726, H727, H740, H865, H1048, H1062, H1334, H1581, H1607, H2122, NCF7, N417, A549, SHP77, H322, NU6-1, H441, CALU1, CALU6, SKLU1, H358, H596, SW900, SKMES1, and ChaGoK-1.

First-strand cDNA synthesis was performed with AMV reverse transcriptase (Boehringer Mannheim, Indianapolis, IN). The resulting cDNA was amplified with primers designed to generate overlapping fragments of the entire *PLK3/PRK* open reading frame (Table 1). Touchdown PCR was performed to amplify the specific fragments in a 10-μl reaction. The final concentrations were as follows: 1X PCR buffer, 1.25 mM $MgCl_2$, 0.2 mM of each dNTP, 1.0 mM of each primer, and 0.25 U AmpliTaq. One hundred nanograms of cDNA were used in each reaction. The cycling conditions were as follows: the DNA was initially denatured at 94°C for 4 min, followed by 20 cycles at 94°C for 30 sec, an annealing temperature 10°C above the calculated T_M and decreasing 0.5°C per cycle for 30 sec, and finally an elongation at 72°C for 90 sec. This was followed by 15 cycles at 94°C for 30 sec, the calculated annealing temperature for 30 sec, and a 72°C extension for 90 sec. The reaction was completed with a final extension at 72°C for 10 min. The annealing temperature was dependent on the primers utilized for amplification. Most primers utilized a calculated T_M between 56°C and 60°C. The PCR products were analyzed on a 2.5% MetaPhor agarose gel stained with 1 μg/ml ethidium bromide and visualized under UV light.

PCR-SSCP analysis of these 40 cell lines uncovered polymorphisms/mutations in nine cell lines (Fig. 1). PCR-SSCP is a polyacrylamide gel-based method that can detect single base changes in DNA due to a mobility shift of a band in the gel (Orita et al., 1989; Murakami et al., 1991; Gaidano

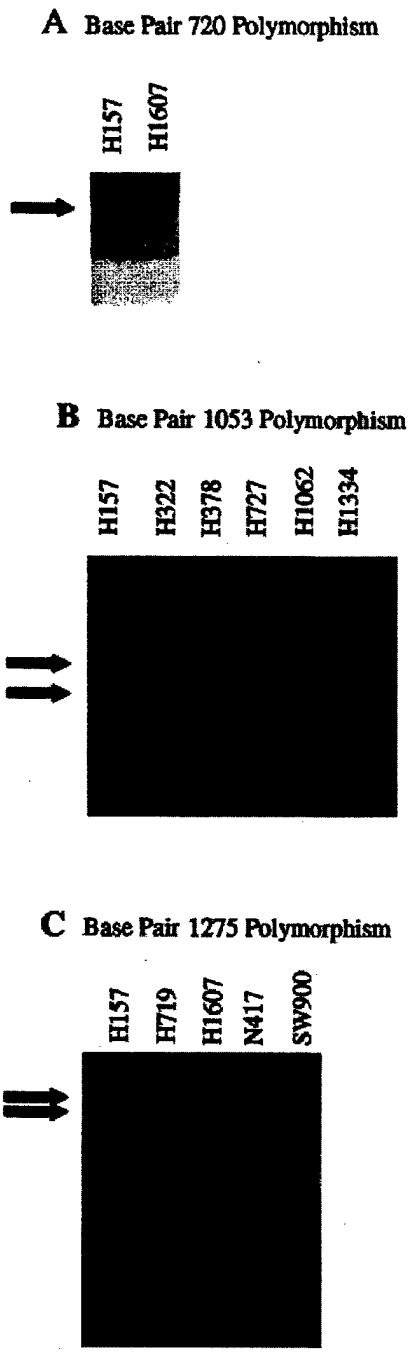

Figure 1. Single-strand confirmation polymorphism/mutation analysis of the *PLK3/PRK* gene in lung tumor cell lines. Samples of the PCR products were separated on 0.5 X MDE gels. One microliter of PCR product was mixed with 9 μl of loading dye and denatured at 94°C for 3 min, then cooled to 4°C. Five microliters were then loaded on the gel. **A:** The region from 718 to 1063 was amplified and analyzed in a 0.5 X MDE gel. Arrow indicates the bands with altered mobility. **B:** The region from 1006 to 1371 was amplified and analyzed on a 0.5 X MDE gel containing 5% glycerol. Arrows indicate the bands with altered mobility. **C:** The region from 1618 to 1902 was amplified and analyzed on a 0.5% MDE gel without glycerol.

et al., 1991). One μl of the amplified product is mixed with 9 μl of a stop solution (95% formamide, 10 mM NaOH, 0.25% bromophenol blue, 0.25% xylene cyanol), denatured in at 94°C for 3 min, and cooled to 4°C. The sample is loaded onto the mutation detection enhancement (MDE) polyacrylamide gel (FMC Bioproducts, Rockland, ME). Two gel conditions were used for each primer set, one gel containing 0.5 X MDE gel and the other gel containing 0.5 X MDE gel plus 5% glycerol. These two gel conditions were used to increase the probability of detecting polymorphisms or mutations. Five μl of the denatured sample were loaded and electrophoresed at 4–6 watts for 12–16 hr in 0.6 X TBE buffer on a DNA sequencing gel apparatus (Gibco/BRL, Gaithersburg, MD). The gel was silver-stained to detect the band shifts following the manufacturer's protocol (Promega, Madison, WI). Briefly, the gel is fixed in 10% acetic acid for 20 min, stained with a silver nitrate solution for 30 min, and developed in a sodium carbonate solution. The reaction is stopped by addition of an equal volume of 10% acetic acid. Altered mobility of a band in the gel, or a band shift, is indicative of a base change in the DNA. The majority of the base changes were detected using a single gel condition (Fig. 1). Polymorphisms/mutations were detected in nine different cell lines at three different locations in the cDNA sequence, and two polymorphisms were detected in one of the cell lines (Table 2).

Each genomic DNA sample containing a band shift was DNA sequenced using a Big Dye terminator reaction mix on an ABI 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). The DNA sequencing reaction contained 100 ng of the PCR product, 3.2 pmol of the appropriate primer, and 8 μl Big Dye terminator reaction mix (Applied Biosystems). The reaction was brought to a final volume of 20 μl with water. The DNA sequencing cycle parameters were as follows: the DNA was denatured at 94°C for 30 sec, the primer was annealed at 50°C for 15 sec, and an extension was at 60°C for 4 min. This cycle was performed 30 times. The sequence reactions were purified with Centri-Sep spin columns, dried in a speed-vacuum, and resuspended in 5:1 deionized formamide to EDTA/Blue dextran loading buffer. The samples were loaded onto a 4.25% polyacrylamide denaturing gel containing 8 M urea. The data were collected and analyzed with the ABI 377 DNA collection software v. 2.5.

The sequence analysis of the PCR products from the cell lines characterized the three distinct poly-

TABLE 2. Mutational Analysis of the *Prk/Plk3* Gene

Cell line	Polymorphism	Location*	Amino acid change	Heterozygosity status
H1607	G to A	720	Silent	Heterozygous
H322	A to G	1053	Silent	Homozygous
H378	A to G	1053	Silent	Homozygous
H727	A to G	1053	Silent	Homozygous
H1062	A to G	1053	Silent	Heterozygous
H1334	A to G	1053	Silent	Heterozygous
H719	G to C	1275	Silent	Heterozygous
H1607	G to C	1275	Silent	Heterozygous
N417	G to C	1275	Silent	Heterozygous
SW900	G to C	1275	Silent	Heterozygous

*Numbering is based on the cDNA sequence of Li et al. (1996).

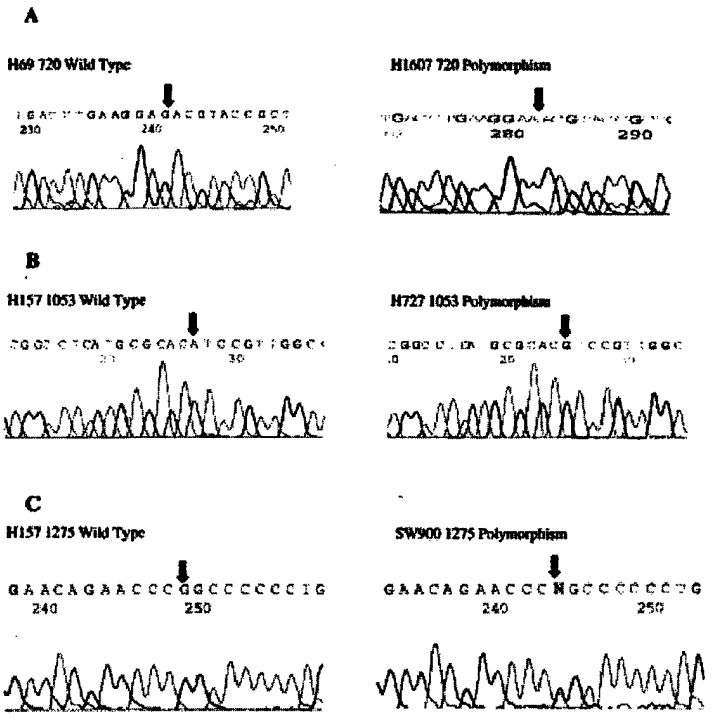

Figure 2. Sequencing of PCR products found to contain mutations by SSCP analysis. The electropherogram for each sequencing reaction is shown, and the base corresponding to the mutation/polymorphism is designated with an arrow over the sequence. The numbering of the sequence is as in Li et al. (1996). The sequencing data suggest that cell lines H1607 and H727 are homozygous for the polymorphism and also that SW900 is heterozygous for the polymorphism at basepair 1275. **A:** H69 wild-type sequence and H1607 polymorphism at basepair 720 showing a G to A polymorphism. **B:** H157 wild-type sequence and H727 polymorphism at basepair 1053 showing an A to G polymorphism. **C:** H157 wild-type sequence and SW900 polymorphism showing a G to C polymorphism.

morphisms (Fig. 2). In all cases, the polymorphisms/mutations did not result in an alteration of the coding sequence (Table 2). Therefore, the silent base changes suggest that these are not somatic mutations, but polymorphisms. Analysis of a series of normal DNA samples would confirm that these polymorphisms occur naturally in the population. While tumor samples demonstrate a decrease in the mRNA levels of *PLK3/PRK* in the lung, the cell lines derived from lung carcinomas showed no mutations in the gene, indicating that mutational inactivation of *PLK3/PRK* may be a rare event in lung tumorigenesis.

To facilitate future mutation screening projects of the *PLK3/PRK* gene, we characterized the intron/exon boundaries (Table 3). If the long-distance PCR (LD-PCR) amplification of normal genomic DNA yielded products with an increased molecular weight compared to the same products generated from cDNA as the template, then it was assumed the product contained the intron sequence. The Advantage cDNA polymerase kit (ClonTech, Palo Alto, CA) was used for these amplifications. A 50-μl reaction was composed of 1X PCR buffer, 500 ng of genomic DNA, 20 μmol of each primer, 0.2 mM of each dNTP, and 1 μl of a

TABLE 3. Intron/Exon Boundaries Found in the *PRK* Gene

Intron	Splice donor[a]	Intron size[b]	Splice acceptor[a]
Intron 1	GTTGGGCAAGgtgggccgagggacgtccgc	166 bp	ctgatgccccctcttcacagGGGGGCTTCG
Intron 2	GCGCGAGAAGgtgggtccaggctcagcggg	88 bp	actccgcgccctcatcgcagATCCTAAATG
Intron 3	CAGCCGAAAGgtaaaggtggtgattcccgc	800 bp*	aggcagttggctctctgcagTCCCTGGCCC
Intron 4	CTCAAGTTGGgtgagactcctgagcctgga	93 bp	gcctctcttgccccatctagGAAATTTTTT
Intron 5	AGAGGAAGAAgtgagttttgaggaaagggg	900 bp*	ctcctccccaccctctttcagGACCATCTGT
Intron 6	GCTGTGTCATgtgagttgcagggtccaggt	99 bp	cccttggccctgccctatagGTACACGCTG
Intron 7	CTTTACCAAGgtctgtggctccccagacct	106 bp	cacatggttcccctccctagGGCTACACCC
Intron 8	AAGAAGAAGAgtgagtctggggtgtcagtg	198 bp	gtatcacctttcaccccagGTAAGAATCA
Intron 9	CAGGCCAGAGgtgaggcgctcaggtggaca	239 bp	ttccctgcccaaccctccagGCTCCAGCAG
Intron 10	AGTGGAGATGgtgaggagccaggcggatgc	137 bp	cagtgccctgtctccttcagGATTTGAAGA
Intron 11	ATGCCCCCAGgtaagggtggggtctggtac	92 bp	tccctcctctcccatgacagCGGAACAGAA
Intron 12	CCAACAGAAAgtaagtgctgttatggggtg	147 bp	cctctgcctccccattctagGACTGTGCAC
Intron 13	CCTCATGAAGgtgtgagggctggggctgtg	570 bp*	gttccagatcctgctcccagGGTGGAGATC
Intron 14	CACTGTCCAGgtaagagcctatccaggagt	107 bp	ctgtcatgctctgtgtgcagGTGAACTTCT

*Approximate size of the intron based on agarose gel electrophoresis.
[a]Capital letters refer to exon sequence and lower-case letters refer to intron sequence.
[b]Size of intron based on DNA sequence data.

50X Advantage cDNA polymerase mix. The cycling parameters were: initial denaturing at 94°C for 4 min, followed by 94°C for 30 sec, 68°C for 6 min for 30 cycles. A final extension at 68°C for 10 min concluded the reaction. Ten microliters of the product was analyzed on a 2.5% Metaphor agarose gel stained with ethidium bromide. The molecular weights of the resulting DNA fragments were compared to a DNA molecular weight marker to estimate sizes. Fragments that differed from their predicted molecular weight based on the cDNA sequence of *PLK3/PRK* were purified and sequenced as described above. Three additional sets of primers were designed for these experiments to generate adequate products for sequence analysis (Table 1).

Sequencing of the LD-PCR products revealed that the open reading frame of the *PLK3/PRK* gene is divided into 15 exons and 14 introns (Table 3). The intron/exon boundaries were characterized by a change of sequence in genomic DNA compared to the cDNA and contained the characteristic gt...ag sequence of introns (Table 3). Most of the 14 introns were small, starting at 88 bp (intron 2) and ranging upwards of 900 bp (intron 5), so that we could get either their complete sequences or an estimation of their size based on the molecular weight markers used in the agarose gels. The approximate size of the *PLK3/PRK* gene based on these results is 7,000 basepairs.

The sequence of exon 1 through intron 2 is a highly GC-rich region (67–73% GC), while the remainder of the gene appears to maintain a GC content near 60% (data not shown). However, this amount of GC content is not predictive of a CpG island (Gardiner-Garden and Frommer, 1987). It should be noted that the predicted 5′ nontranslation sequence is also very GC-rich (Li et al., 1996). In fact, these sequences were also conserved in a murine counterpart, *Fnk* (Donahue et al., 1995). As pointed out by Chase et al. (1998), the deduced amino acid sequence of *PLK3/PRK* may lack a short fragment of coding sequence, as described in the murine counterpart. One possible reason for failure to obtain the 5′ coding sequences during RACE or library screening is the presence of the high GC content.

Checkpoint failure is a key mechanism of tumorigenesis. Previous research has identified and characterized checkpoint genes such as *TP53* and *ATM*, and functional loss of these gene products, due to mutations or other structural abnormalities, often leads to malignant transformation. We have reported that *PLK3/PRK* expression is downregulated in a majority of lung and head/neck carcinoma samples examined (Li et al., 1996). In the current study, we show that no missense or nonsense mutations were detected in lung-tumor-derived cell lines. *PLK3/PRK* is also downregulated in HNSCC, suggesting that compromised expression of *PLK3/PRK* is correlated with the development of malignancy. Our FISH study localized the *PLK3/PRK* gene to 8p21. This chromosomal locus undergoes loss of heterozygosity in many human cancers, including HNSCC and lung carcinomas (Dai et al., 2000), and appears to harbor two putative tumor suppressor genes (Imbert et al., 1996). Considering that constitutive expression of *PLK3/PRK* signifi-

cantly suppresses the proliferation of transformed lung fibroblast cells, it is tempting to speculate that the *PLK3/PRK* gene may play an important role in regulating the rate of cell proliferation.

In this study, we screened the *PLK3/PRK* gene for mutations because of its interactions in controlling the cell cycle, its location in a region of frequent loss of heterozygosity in lung cancer, and because it is downregulated in head and neck tumors (Conn et al., 2000; Dai et al., 2000). The elucidated intron/exon boundaries of the *PLK3/PRK* gene are reported. The gene structure was found to contain 15 exons and 14 introns ranging in size from 88–900 basepairs. Mutations/polymorphisms in the *PLK3/PRK* gene were identified through SSCP analysis and DNA sequencing in 40 lung carcinoma cell lines. No missense or nonsense mutations were found in the cell lines examined. However, three polymorphisms in the coding sequence were identified: a G to A at position 720, an A to G at 1053, and a G to C at 1275. Together, these data suggest that inactivation of *PLK3/PRK* by somatic mutation is a rare event in lung tumorigenesis.

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Cell Cycle Arrest and Apoptosis Induced by Human Polo-Like Kinase 3 Is Mediated through Perturbation of Microtubule Integrity

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Human Polo-like kinase 3 (Plk3, previously termed Prk or Fnk) is involved in regulation of cell cycle progression through the M phase (B. Ouyang, H. Pan, L. Lu, J. Li, P. Stambrook, B. Li, and W. Dai, J. Biol. Chem. 272:28646–28651, 1997). Here we report that in most interphase cells endogenous Plk3 was predominantly localized around the nuclear membrane. Double labeling with Plk3 and γ-tubulin, the latter a major component of pericentriole materials, revealed that Plk3 was closely associated with centrosomes and that its localization to centrosomes was dependent on the integrity of microtubules. Throughout mitosis, Plk3 appeared to be localized to mitotic apparatus such as spindle poles and mitotic spindles. During telophase, a significant amount of Plk3 was also detected in the midbody. Ectopic expression of Plk3 mutants dramatically changed cell morphology primarily due to their effects on microtubule dynamics. Expression of a constitutively active Plk3 (Plk3-A) resulted in rapid cell shrinkage, which led to formation of cells with an elongated, unsevered, and taxol-sensitive midbody. In contrast, cells expressing a kinase-defective Plk3 ($Plk3^{K52R}$) mutant exhibited extended, deformed cytoplasmic structures, the phenotype of which was somewhat refractory to taxol treatment. Expression of both Plk3-A and $Plk3^{K52R}$ induced apparent G_2/M arrest followed by apoptosis, although the kinase-defective mutant was less effective. Taken together, our studies strongly suggest that Plk3 plays an important role in the regulation of microtubule dynamics and centrosomal function in the cell and that deregulated expression of Plk3 results in cell cycle arrest and apoptosis.

Members of the Polo family of protein kinases, conserved through evolution, have been described in yeast (15), *Caenorhabditis elegans* (25), *Drosophila melanogaster* (8), *Xenopus laevis* (16) mouse (7, 27), and human (12, 18). The founding member of this family, Polo, was originally identified in the fruit fly and was shown to be a serine-threonine kinase that is required for mitosis (8). Mutations in the Polo gene result in abnormal mitotic and meiotic division (10). The kinase activity of Polo peaks cyclically at anaphase-telophase, and the protein also undergoes translocations during the cell cycle progression (9). Whereas it is located predominantly in the cytoplasm during interphase, at M phase it becomes associated with condensed chromosomes and other components of the mitotic apparatus, including centrosomes, kinetochores, and the spindle midzone (9). In addition to the conserved kinase domain, Polo family proteins all share a short amino acid sequence termed the Polo box (18). Mutations in the Polo box of the Plk homologue in budding yeast (Cdc5) that do not affect kinase activity abolish the ability of this protein to complement functionally temperature-sensitive mutants of budding yeast (30), suggesting that the Polo box is essential for its biological activity.

Mammalian cells contain at least three Polo family proteins (human Polo-like kinase 1 [Plk1], Plk2, and Plk3) that exhibit marked sequence homology to *Drosophila* Polo (7, 13, 18, 27). As cells progress through the cell cycle, Plk proteins undergo substantial changes in abundance, kinase activity, or subcellular localization. In human cells, the levels of Plk1 protein and its kinase activity peak at mitosis (13). During mitosis, Plk1 transiently associates with mitotic structures such as the spindles, kinetochores, and centrosomes (1, 11). Recent studies have shown that Plk1 contributes to a variety of mitotic (or meiotic) events, including activation of cyclin B-Cdc2 (CDK1), breakdown of the nuclear membrane, centrosome maturation, and formation of the bipolar spindle at the onset of mitosis (4, 22, 26).

Plk3, originally cloned and characterized in our laboratory, appears to function differently from Plk1. The abundance of Plk3 remains relatively constant during the cell cycle, and its kinase activity peaks during late S and G_2 phases (24). Furthermore, Plk3 phosphorylates Cdc25C on serine-216, resulting in inhibition of the activity of this protein (23), whereas phosphorylation of cyclin B by Plk1 results in its translocation from the cytosol to the nucleus, thus activating Cdc2 kinase (31). In addition, the amount of Plk3 mRNA but not of Plk1 mRNA is rapidly and transiently increased in response to mitogenic stimulation (18). Plk2 (also known as Snk) was originally identified as the product of an immediate-early gene and is less characterized than are Plk1 and Plk3. Recent studies have shown that both Snk and Fnk associate with CIB, a calmodulin-related protein (14). Both Snk and Fnk have been implicated in long-term synaptic plasticity and thus may perform postmitotic functions (14).

The present study was designed to reveal the subcellular localization of Plk3 during the cell cycle, describe its potential

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role in regulating microtubule dynamics and the microtubule organization center (MTOC), and evaluate its role in the maintenance of cell morphology, viability, and malignant transformation.

MATERIALS AND METHODS

Cell culture and treatment. The GM00637 cell line (human fibroblast) was originally obtained from the Coriell Institute for Medical Research. A549 (lung carcinoma), HeLa (cervical carcinoma), Daudi (lymphoblastic leukemia), HEL (erythroleukemia), HL-60 (myelogenous leukemia), PC-3 (prostate carcinoma), LNCaP (prostate carcinoma), and DU145 (prostate carcinoma) cell lines were obtained from the American Type Culture Collection. Cells were cultured in culture dishes or on Lab-Tek II chamber slides (Fisher Scientific) in appropriate media supplemented with 10% fetal bovine serum and antibiotics (100 μg of penicillin and 50 μg of streptomycin sulfate per ml) with 5% CO_2. For some experiments, A549 cells at about 80% confluence were treated with a low temperature (4°C) for 5 min or taxol (5 μM) for 4 h before analysis (unless specified otherwise).

Transfection. A549 cells were transfected, using the Lipofectamine method, with pCR259–Plk3-A, pCR259-Plk3^{K52R}, or the empty vector (GIBCO/Invitrogen). The constitutively active Plk3 cDNA was as described previously (18). The Plk3^{K52R} was obtained by replacing the conserved lysine-52 residue with arginine; the resulting protein was kinase defective (23).

Immunoblotting and pull-down assays. Cultured cells were lysed as described earlier (24). Equal amounts (50 μg) of protein lysates and purified Plk1 and Plk3 were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies to human Plk3 (Pharmingen [23]), BUBR1, α-tubulin (Sigma), Plk1 (Zymed), or cyclin B (DAKO). Recombinant His_6-Plk3 expressed with the use of a baculoviral expression system as described earlier (23) was affinity purified with and subsequently conjugated to Ni-nitrilotriacetic acid resin (Qiagen). Flag-tagged Plk1 was a gift from John Cogswell (GlaxoSmithKline). Specific signals were detected with horseradish peroxidase-conjugated goat secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Fluorescence microscopy. Localization of Plk3 was determined by double immunofluorescence analysis of a centrosomal marker. Cells were quickly washed with phosphate-buffered saline (PBS) and fixed in methanol for 5 min at room temperature. Fixed cells were treated with 0.1% Triton X-100 in PBS for 5 min and were then washed three times with ice-cold PBS. After blocking with 2.0% bovine serum albumin (BSA) in PBS for 15 min on ice, cells were incubated for 1 h with mouse monoclonal Plk3 immunoglobulin G (IgG, 4 μg/ml) and/or rabbit anti-γ-tubulin IgG (T3559, 1:250 dilution; Sigma) in 2% BSA solution, washed with PBS, and then incubated with rhodamine red X-conjugated anti-mouse IgGs and/or fluorescein isothiocyanate-conjugated anti-rabbit IgGs (Jackson ImmunoResearch) at 4°C for 1 h in the dark. Cells were finally stained with 4′,6′-diamidino-2-phenylindole (DAPI) (1 μg/ml; Fluka) for 5 min. Fluorescence microscopy was performed on a Nikon microscope, and images were captured using a digital camera (Optronics) using Optronics MagFire and Image-Pro Plus softwares.

Flow cytometry. A549 cells transfected with or without Plk3 mutants for 14 h were analyzed for cell cycle stage and apoptosis. Briefly, cells were fixed with methanol, treated with Triton X-100 (0.25% in PBS) for 5 min, washed with PBS with 1% BSA, and incubated for 1 h with (or without as a control) an antibody to a caspase-cleaved poly(ADP-ribose) polymerase fragment (PARP p85) (Promega). Cells were rinsed with PBS and then incubated with a second antibody conjugated with fluorescein isothiocyanate for 1 h, rinsed with PBS containing 1% BSA, and stained with DAPI (1 μg/ml). The fluorescence of cells processed for flow cytometry was measured by the ELITE.ESP cytometer/cell sorter (Coulter) as described previously (19). Each experiment was repeated at least three times.

RESULTS

Although early experiments have shown that our Plk3 antibody does not cross-react with Plk1 (32), we further determined the specificity of the Plk3 antibody via Western blotting. Two sets of purified recombinant Plk1 and Plk3 samples, as well as A549 cell lysates, were blotted with antibodies to Plk1 and Plk3, respectively. Figure 1 shows that neither Plk1 (Fig. 1A) nor Plk3 (Fig. 1B) antibody cross-reacted with each other. To further test the antibody, we examined expression of Plk3 in various cell lines via Western blotting. Figure 1C showed that the Plk3 antibody detected a single antigen that migrated at the position predicted for full-length Plk3. No additional prominent band(s) was detected by the antibody in these cell lines, indicating that our Plk3 antibody did not cross-react with Plk1. As a loading control, the same blot was stripped and reprobed with an antibody to BUBR1 (Fig. 1D), a spindle checkpoint gene product.

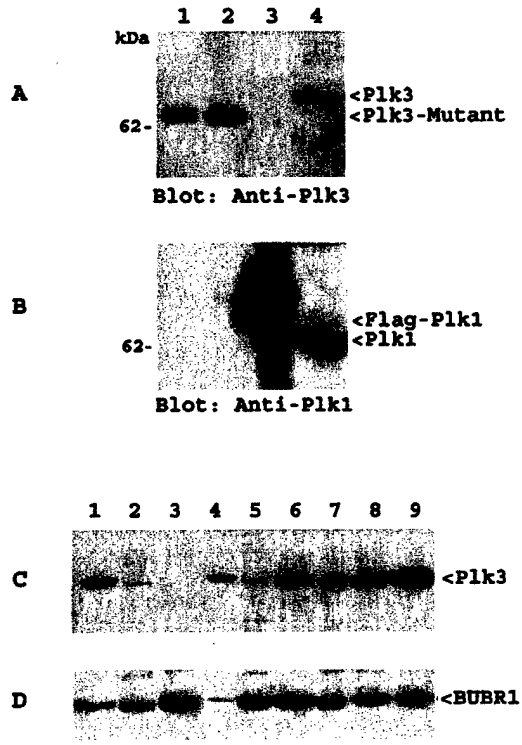

FIG. 1. Plk3 antibody did not recognize Plk1. (A) Purified recombinant His_6–Plk3-A (lanes 1 and 2) and Flag-Plk1 (lane 3), as well as A549 cell lysates (lane 4), were blotted with the anti-Plk3 antibody. Recombinant Plk3 migrated faster than the cellular form because of a short deletion at the amino terminus (20). (B) A duplicate blot as shown in panel A was blotted for Plk1. Recombinant Plk1 migrated slightly more slowly than the cellular form because of an addition of the Flag tag. (C and D) Protein lysates (50 μg/lane) from A549, DAMI, Daudi, DU145, GM00637, HEL, HeLa, LNCaP, and PC-3 were blotted with the Plk3 antibody (C) or BUBR1 antibody (D).

In an attempt to identify potential physiological targets of Plk3, we examined the subcellular localization of Plk3 in cultured cell lines via indirect immunofluorescence microscopy. We detected (Fig. 2A) strong Plk3-specific staining as spots around the nuclear membrane of A549 cells, although diffuse, much weaker staining was also detected in the cytoplasm. Perinuclear, condensed spots were detected in over 90% of the cells. When the spot was detected in cells, those cells usually contained one spot (69%). This Plk3 subcellular localization pattern was also observed in the other two cell lines (GM00637 and HeLa), indicating a common cellular function associated with Plk3 subcellular localization. Double-labeling experiments using an antibody to γ-tubulin, a major component of pericentriole materials, showed that Plk3 localized around cen-

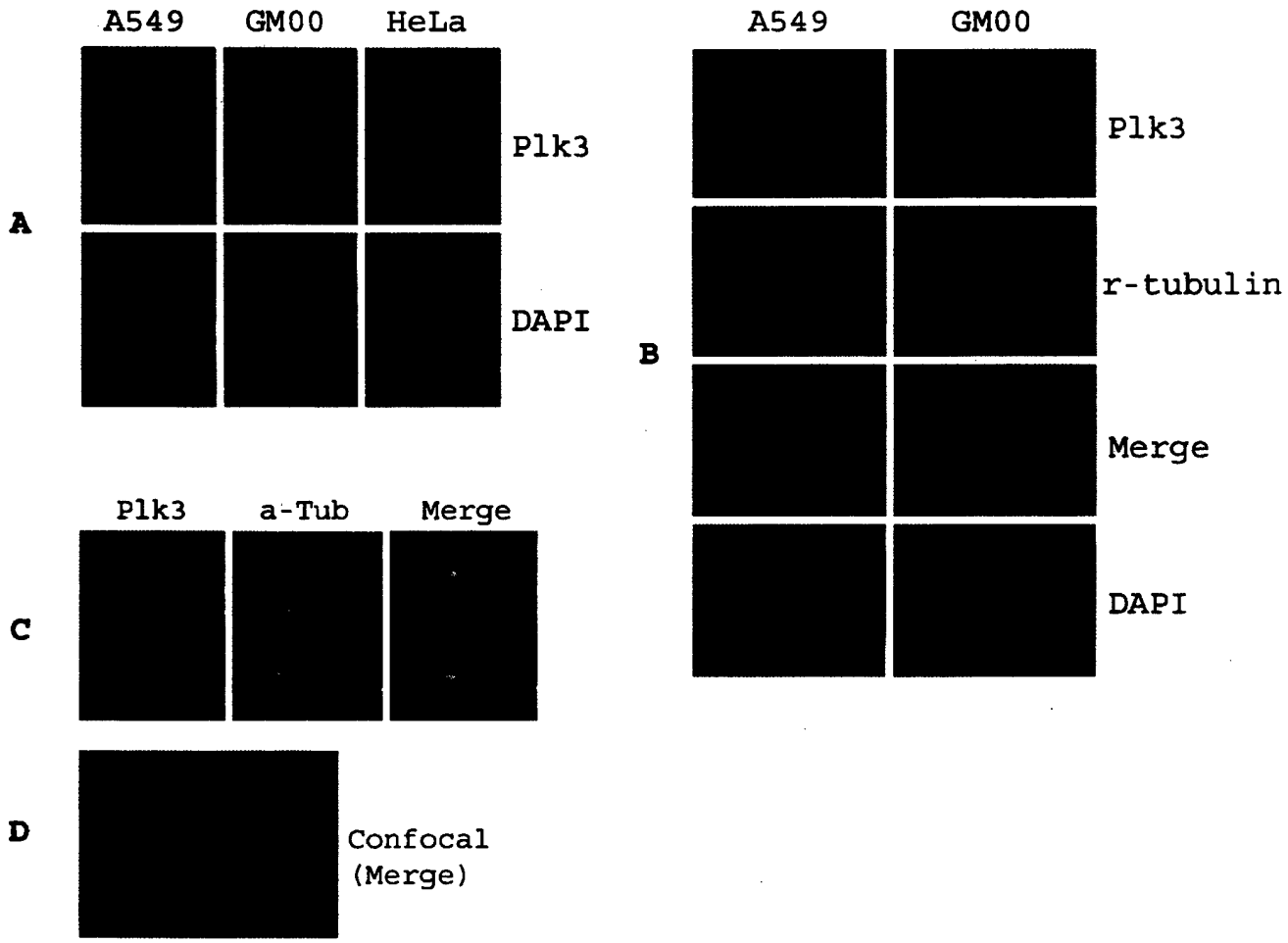

FIG. 2. Plk3 colocalized with centrosomes. (A) A549, GM00637, and HeLa cells cultured on chamber slides were incubated with an antibody to Plk3, and DNA was stained with DAPI. Plk3 signals were detected by indirect immunofluorescence microscopy. (B) A549 and GM00637 cells were double stained with antibodies to Plk3 (red) and γ-tubulin (green), a centrosome-specific marker. (C) A549 cells were double stained with antibodies to Plk3 (red) and α-tubulin (green). (D) A549 cells were triple stained with Plk3 (red), α-tubulin (green), and DAPI (blue). Specific signals were detected by confocal microscopy. Arrowheads indicate the location of MTOCs.

trosomes in both A549 and GM00637 cells (Fig. 2B). However, the distribution of Plk3 staining was slightly more diffuse than that of γ-tubulin. The same colocalization of Plk3 with centrosomes was observed in several other cell lines, including HeLa cells (data not shown). Double staining using an antibody to α-tubulin confirmed that Plk3 was tightly associated with MTOC (Fig. 2C and D, arrowheads).

Interestingly, the expression of Plk3 in some cells was more extended and diffuse than were the discrete spots as described above (Fig. 3A). A close examination revealed that these cells contained duplicated centrosomes as shown by γ-tubulin staining (Fig. 3A, arrowheads). Plk3 thus appeared to migrate with the duplicated centrosomes. During prophase, a significant amount of Plk3 was detected around spindle pole regions (Fig. 3B). As mitosis progressed, Plk3 colocalized with mitotic spindles during metaphase. By telophase, concentrated Plk3 was found at the midbody region as well as at spindle poles (Fig. 3B). It should be noted that low levels of Plk3 were also detected in the other regions of the cell during telophase.

Subcellular localization patterns suggest that Plk3 may be involved in regulating the activity of MOTC and/or microtubule dynamics. To determine whether disruption of Plk3 function would affect microtubule integrity and cell viability, we transfected A549 cells with a plasmid construct expressing constitutively active Plk3 (Plk3-A) (23). As a control, a kinase-defective Plk3 mutant ($Plk3^{K52R}$) expression construct was also transfected. Twenty hours posttransfection, cells were first analyzed for expression of transfected Plk3 mutants. Figure 4A shows that both $Plk3^{K52R}$ and Plk3-A were expressed at similar levels (Fig. 4A, lanes 3 and 4, arrowhead Plk3-Mutant). The transfected Plk3 mutant proteins were highly expressed compared with the endogenous Plk3 (arrow Plk3). The faster mobility of transfected Plk3 mutant proteins was due to a short deletion at the amino terminus (18). Fluorescence microscopy revealed (Fig. 4B) that a significant fraction (about 20%) of the transfected cells expressed high levels of Plk3-A or $Plk3^{K52R}$. Cells expressing Plk3-A appeared shrunken and spherical (Fig. 4B and Table 1). On the other hand, most cells expressing $Plk3^{K52R}$ remained attached, exhibiting a variety of extended, irregular shapes. Endogenous Plk3 signals were usually over-

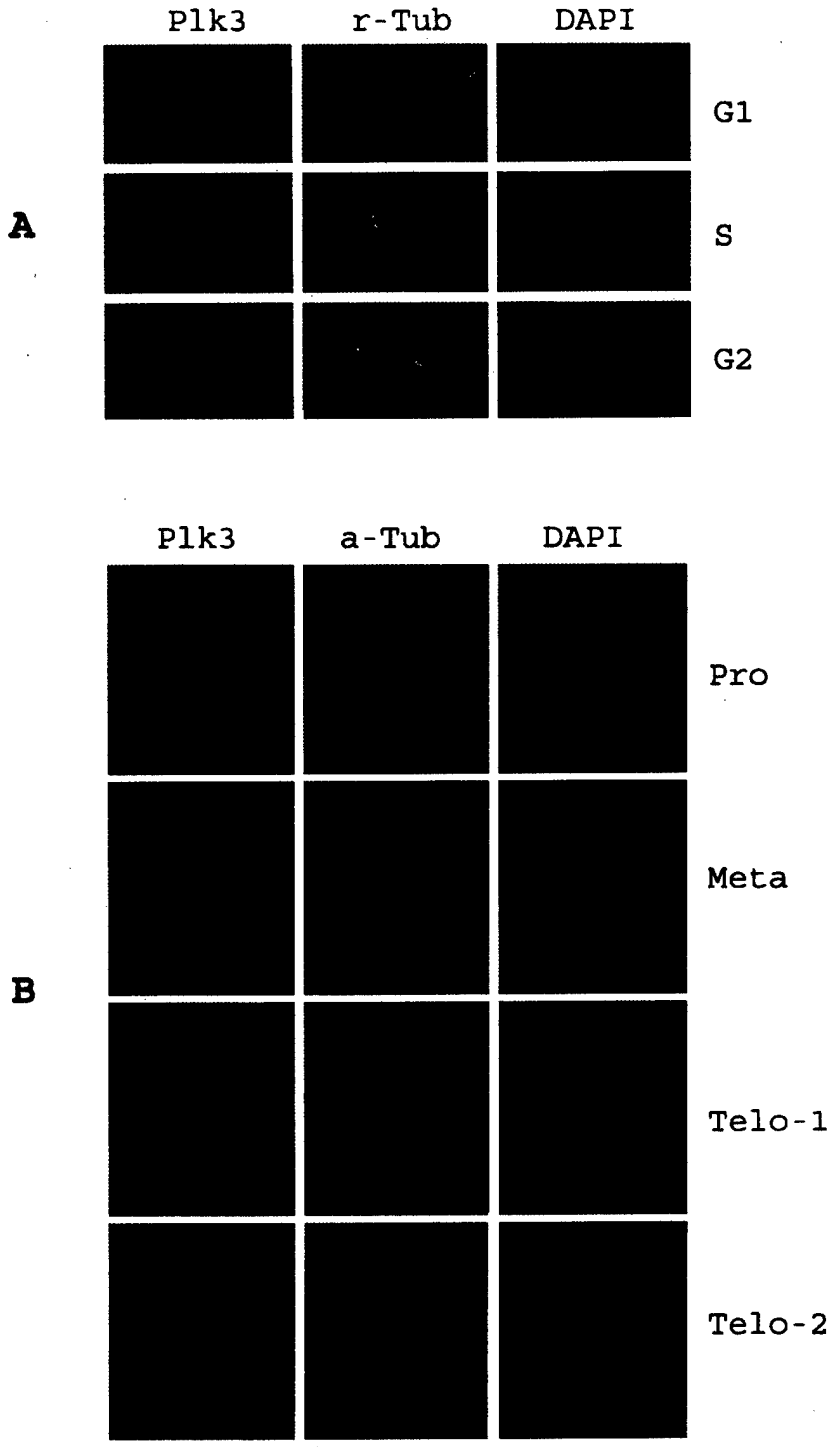

FIG. 3. Plk3's association with centrosomes/spindle poles was dependent on cell cycle status. (A) A549 cells were stained with antibodies to Plk3 (red) and γ-tubulin (r-tubulin) (green). Various cell cycle stages were determined by DNA staining, as well as the number and position of centrosomes (arrowheads) in the cells. G_1 phase with unduplicated centrosome; S phase with duplicated centrosomes; G_2 phase with duplicated and physically separated centrosomes and with intact nuclear membrane. (B) A549 cells were stained with antibodies to Plk3 (red) or α-tubulin (a-Tub) (green). Cells of various mitotic stages as shown by the DNA staining were presented.

whelmed by the strong signals of ectopically expressed Plk3 mutant proteins, although sometimes bright dots of endogenous Plk3 at MTOC could also be recorded along with ectopically expressed Plk3-A during imaging (Fig. 4C).

Frequently, two cells that expressed Plk3-A were found together often attached to each other (Fig. 5A). In fact, over 80% of the cells expressing Plk3-A either formed doublets or were near each other (Table 2). Occasionally, four Plk3-A-

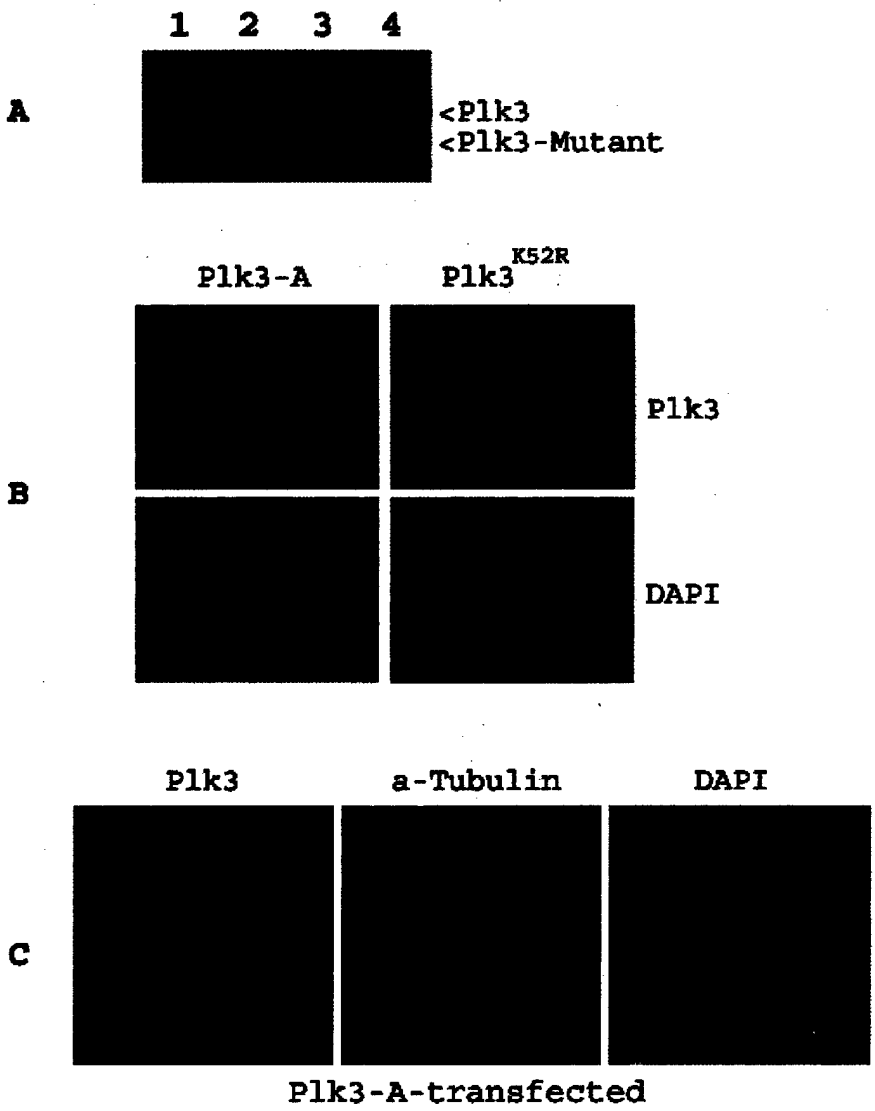

FIG. 4. Ectopic expression of Plk3 mutants. (A) Protein lysates from the cells transfected for 20 h with Plk3-A (lane 3) and Plk3^{K52R} (lane 4) expression constructs, as well as the vector (lane 2), were blotted for Plk3. Protein lysates from untransfected cells were also used as a control (lane 1). The endogenous (arrowhead Plk3) protein and transfected (arrowhead Plk3-Mutant) Plk3 mutant protein are shown. (B) A549 cells transfected with Plk3-A or Plk3 ^{K52R} were stained with the Plk3 antibody (red) and DAPI (blue). (C) A549 cells transfected with Plk3-A were stained with antibodies to Plk3 (red) and α-tubulin (green). DNA was stained with DAPI (blue). Besides six cells expressing high levels of transfected Plk3-A, the endogenous Plk3 concentrated at the centrosomal regions was also seen.

expressing cells were stuck together (Fig. 5B). These cells were spherical and loosely attached to the culture plates and resembled mitotic cells. Double-labeling experiments with an antibody to α-tubulin revealed that these cells were connected by an elongated and unsevered midbody (Fig. 5A, arrowheads), suggesting that they were incapable of completion of cytokinesis. The apparent defect in cytokinesis of those Plk3-A-expressing cells eventually resulted in apoptosis, as evidenced by chromatin condensation and nuclear fragmentation (Fig. 5C, arrowheads).

In contrast, cells expressing Plk3^{K52R} typically formed extended cytoplasmic protrusions or branches (Fig. 5D, 12 h), which in turn formed cytoplasmic bodies (arrowheads). These protrusions/bodies also contained microtubule (α-tubulin staining) structures. As Plk3^{K52R} continued to exert its effect, most cells contained granules that were brightly stained with the Plk3 antibody. Eventually, some Plk3^{K52R}-expressing cells appeared to fragment their cytoplasmic extensions and underwent apoptosis as shown by highly condensed, hyperchromatic chromatin and fragmented nuclei (Fig. 5D, 36 h, arrowheads).

TABLE 1. Percentage of round cells expressing Plk3-A or Plk3^{K52R} at various times after transfection[a]

Treatment	% At different times		
	14 h	24 h	36 h
Plk3-A	90	90	97
Plk3^{K52R}	34	26	27

[a] Transfected A549 cells were labeled with the antibody to Plk3 and examined by fluorescence microscopy. At least 200 cells expressing transfected Plk3 mutant proteins were counted for each time point, and data were summarized from two independent experiments.

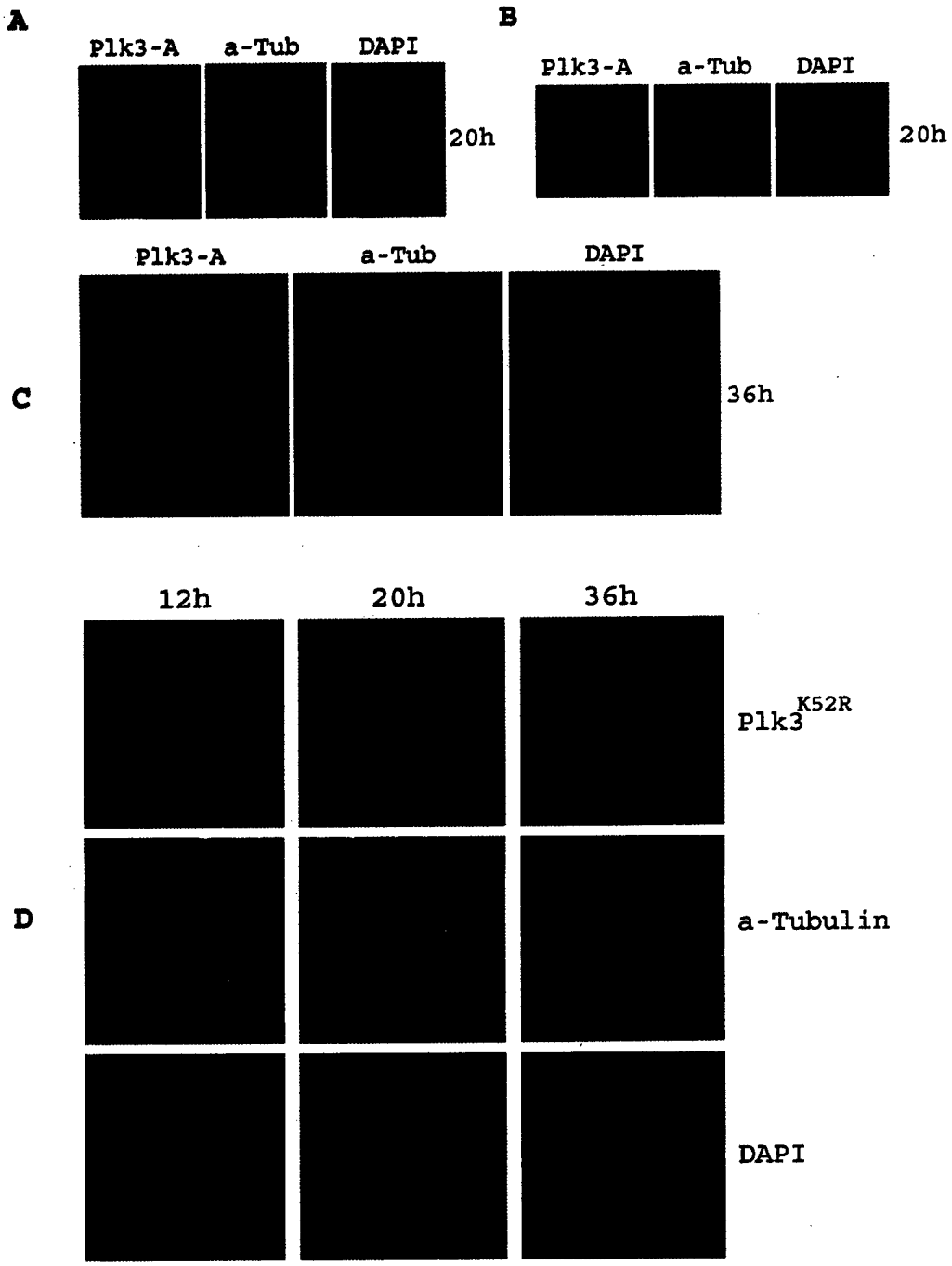

FIG. 5. Expression of Plk3 mutants resulted in a dramatic change in cell shape. (A) A549 cells transfected with Plk3-A for 20 h were stained with antibodies to Plk3 (red) and α-tubulin (green). Arrowheads indicate the position of midbodies. (B) An example of four cells stuck together that were transfected with Plk3-A for 20 h. (C) A549 cells transfected with Plk3-A expression construct for 36 h were stained with antibodies to Plk3 (red) and α-tubulin (green). Arrowheads indicate Plk3-A-expressing cells undergoing apoptosis. (D) A549 cells transfected with $Plk3^{K52R}$ expression construct for various times were stained with antibodies to Plk3 (red) and α-tubulin (green). Green arrowheads indicate the cytoplasmic bodies. White arrowheads indicate the apoptotic cells, and red arrowheads show the deformed nuclei.

The cytoplasmic distortion in the $Plk3^{K52R}$-overexpressing cells also exerted apparent stress on nuclei, resulting in their elongated or deformed shape (DAPI staining, red arrowheads).

To further link Plk3 to microtubule dynamics, we next tested whether centrosomal localization of endogenous Plk3 was affected by microtubule depolymerization. Compared with the untreated controls, exposure of A549 cells to a low temperature (4°C) resulted in diffusion of Plk3 (Fig 6A). Plk3 spread throughout the cell, whereas centrosome integrity (γ-tubulin staining) was largely unaffected. A subsequent analysis of cells treated with nocodazole confirmed that, when microtubule in-

TABLE 2. Percentage of doublets with midbody that express Plk3-A at various times after transfection in presence or absence of taxol[a]

% Found at different times with and without taxol					
14 h (no taxol)	24 h (no taxol)	36 h (no taxol)	14 h (taxol)	24 h (taxol)	36 h (taxol)
83	79	64	3.8	2.8	*

[a] Transfected A549 cells were labeled with the antibody to Plk3 and examined by fluorescence microscopy. At least 200 cells expressing transfected Plk3-A were counted for each time point, and data were summarized from two independent experiments. *, cells became apoptotic.

tegrity was disrupted, Plk3 was no longer localized around centrosomes as the discrete spots (data not shown). Interestingly, taxol, which causes stabilization of the microtubule, did not significantly affect the localization of endogenous Plk3 to MTOC. Whereas control cells formed microtubule bundles upon taxol treatment, cells expressing transfected $Plk3^{K52R}$ were capable of forming protrusions (spikes) (Fig. 6B, arrowheads), although they were not as extended as in the non-taxol-treated ones (Fig. 5D). In contrast, cells expressing Plk3-A formed thick microtubule bundles. Midbody-like structures were observed rarely, if at all, in Plk3-A-expressing cells treated with taxol (Fig. 6B and Table 2). The mechanism by which taxol induces disappearance of the midbody by taxol is unclear at present.

To examine the effect of Plk3 mutant expression on cell cycle status, A549 cells transfected with $Plk3^{K52R}$, Plk3-A, or vector alone for 12 h were stained with DAPI and their DNA content was analyzed by flow cytometry. Figure 7 shows that transfection of A549 cells with the vector alone did not alter the cell cycle distribution compared with that of parental cells. How-

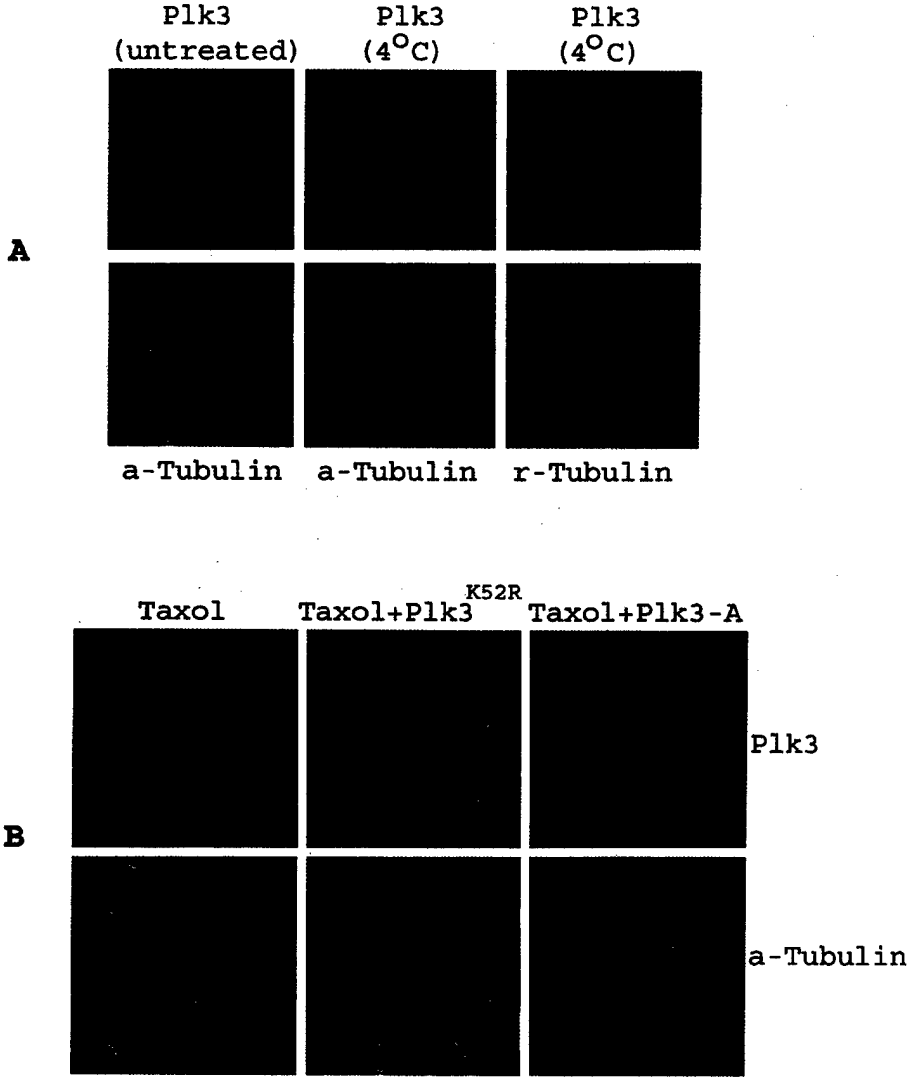

FIG. 6. Plk3's localization to centrosomes is sensitive to cold but not to taxol. (A) A549 cells treated with or without cold were stained with antibodies to Plk3 (red) and α-tubulin (a-Tubulin) (green) or γ-tubulin (r-Tubulin) (green). (B) Parental A549 cells or cells transfected with Plk3-A or $Plk3^{K52R}$ in the presence of taxol (5 μM, 16 h) were stained with Plk3 (red) and α-tubulin (green). Arrowheads indicate the cytoplasmic protrusions in a cell expressing high levels of $Plk3^{K52R}$.

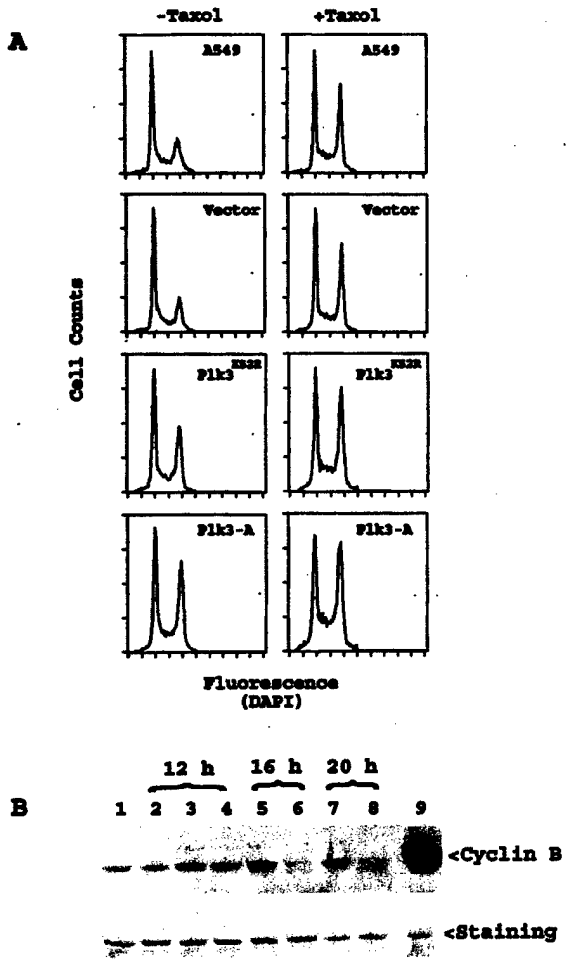

FIG. 7. Mitotic arrest induced by Plk3-A and Plk3^{K52R}. (A) A549 cells transfected with vector (pCR259), Plk3^{K52R}, or Plk3-A expression constructs for 12 h were treated with or without taxol (25 μM) for an additional 4 h. The parental and transfected A549 cells were stained with DAPI, and their cell cycle status was analyzed by flow cytometry. (B) A549 cells transfected with vector (lane 2), Plk3^{K52R} (lanes 3, 5, and 7), or Plk3-A (lanes 4, 6, and 8) for various times as indicated were lysed, and equal amounts of proteins were blotted for cyclin B expression. A549 cells treated with (lane 9) or without (lane 1) nocodazole were used as controls.

ever, expression of Plk3-A for 12 h significantly elevated the proportion of G_2/M cells. Expression of Plk3^{K52R} also increased the frequency of G_2/M cells, albeit to a lesser degree than that of Plk3-A (Fig. 7A and Table 3). Taxol treatment also resulted in a mitotic arrest (Fig. 7A and Table 3). Compared with that of taxol alone, a significant reduction in the G_1 population coupled with an increase in the S and G_2/M populations was observed in cells transfected with Plk3-A or Plk3^{K52R} in the presence of taxol (Table 3), indicating that the effect of taxol and Plk3 on cell cycle arrest was additive.

As an alternative method to confirm mitotic arrest after expression of Plk3-A and Plk3^{K52R}, we measured cyclin B levels. Western blotting analysis showed (Fig. 7B) that significantly more cyclin B was detected in cells expressing Plk3-A and Plk3^{K52R} than in control cells 12 h posttransfection. Interestingly, by 16 h (or longer) posttransfection, cyclin B was rapidly decreased in cells transfected with Plk3-A (Fig. 7B). On the other hand, no significant cyclin B degradation was observed in cells expressing Plk3^{K52R} until at least 16 h posttransfection. The disappearance of cyclin B suggested that cells either exited from mitosis or underwent apoptosis. Further analysis revealed (Fig. 8) that, whereas few parental A549 or vector-transfected cells contained cleaved PARP p85 fragment, an early apoptotic marker, a significant fraction of Plk3-A-transfected cells were PARP p85 positive (Fig. 8, arrowhead), indicating that enforced expression of Plk3-A initiated programmed cell death following mitotic arrest. Consistent with cell cycle arrest and cyclin B levels (Fig. 7), expression of Plk3^{K52R} also resulted in a significant increase in the number of cells expressing PARP p85 (Fig. 8).

DISCUSSION

Polo-like kinases play important roles in the regulation of the G_2/M transition, mitotic progression, and DNA damage checkpoint response (22, 28, 32). To date, much effort has been focused on the role of Plk1 during cell cycle control in vertebrates. We have been studying the biological role of human Plk3, and our early studies have indicated that Plk3 functions differently from Plk1 (23, 32). Here we report that Plk3 colocalizes with centrosomes or the spindle poles. Several previous studies show that Plk1 also localizes to centrosomes (11) and the kinetochore/centromere (1). Thus, Plk3 shares a subcellular localization pattern (Fig. 2 and 3) similar to that of Plk1 during interphase and mitotic prophase (11). However, Plk3 colocalizes with the mitotic spindle (Fig. 3), whereas Plk1 is confined to the spindle poles during metaphase (11). Plk3 also differed from Plk1 (1, 11) in that no significant Plk3 signals were detected on kinetochores. In all stages of the cell cycle, Plk3 is apparently associated with the centrosome(s) or the spindle poles, strongly suggesting a role for Plk3 in the regulation of MTOC as well as mitosis. Plk3 is not a core component of centrioles, because its subcellular localization is much more diffuse than that of γ-tubulin (Fig. 2). In addition, its association with MTOC is microtubule dependent, as depolymerization of microtubules with cold treatment significantly compromised the localization of Plk3 to the centrosomal regions (Fig. 6).

TABLE 3. Cell cycle distribution (%) of A549 transfected with or without various expression constructs[a]

Treatment	% Found at different phases		
	G_1	S	G_2/M
A549	43	38	19
Vector	47	36	18
Plk3-A	33	36	32
Plk3^{K52R}	38	35	28
A549 + taxol	35	39	26
Vector + taxol	34	35	30
Plk3-A + taxol	26	40	33
Plk3^{K52R} + taxol	29	38	33

[a] Parental A549 cells or A549 cells transfected with Plk3-A, Plk3^{K52R}, or the vector alone for 12 h were treated with or without taxol for an additional 4 h. The cells were then processed and stained with propidium iodide. Cell cycle distributions were analyzed by flow cytometry. Data were summarized from three independent experiments.

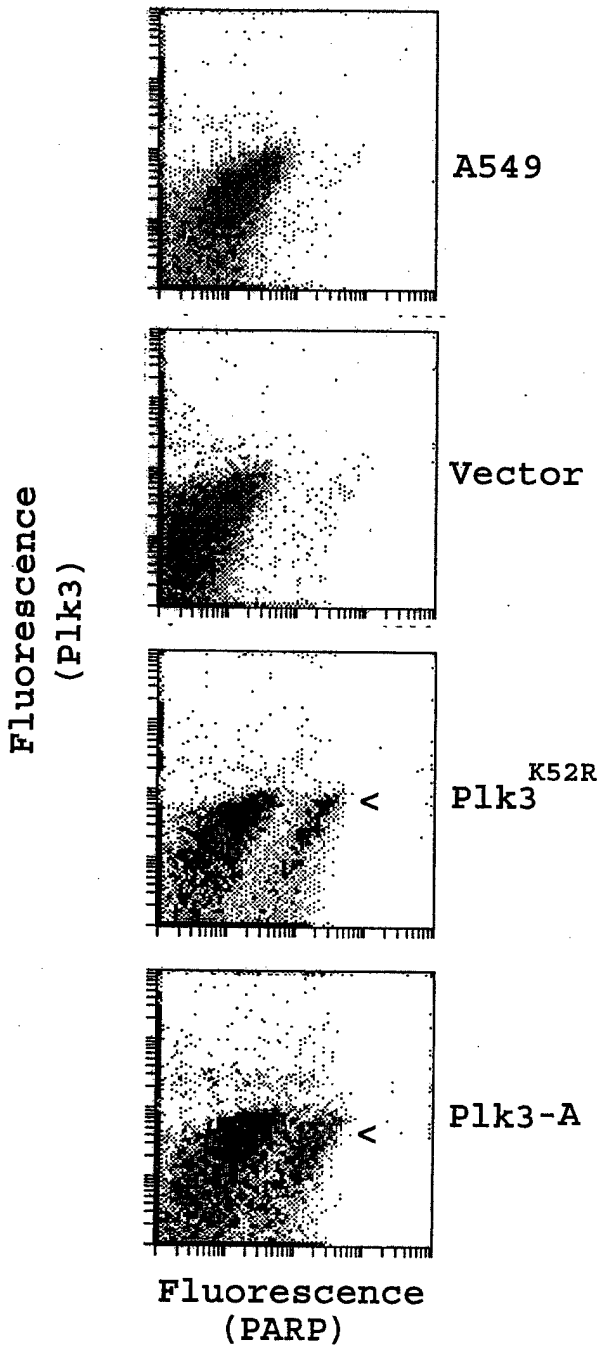

FIG. 8. Apoptosis induced by Plk3-A and Plk3^{K52R}. A549 cells transfected with vector (pCR259), Plk3^{K52R}, or Plk3-A expression constructs for 16 h were stained with antibodies to Plk3 and to caspase-cleaved PARP p85 fragment, an early apoptotic marker. The fluorescence of the cells was then analyzed by flow cytometry. Parental A549 cells were processed and analyzed in the same manner as a control.

Antibody injection studies suggest that Plk1 may be involved in centrosome separation and maturation (17). However, Plk1 is rapidly degraded upon exit from mitosis by the antigen-presenting-cell-mediated proteolytic pathway (21). As a result, the Plk1 protein level remains low during interphase. Its level increases rapidly at the onset of mitosis, indicating that Plk1 functions primarily during mitosis (6, 22). It appears that no rigorous tests were performed in these studies to assure that the antibodies used did not cross-react with Plk3; it is possible that the proposed Plk1 functions in centrosome separation and that maturation may be partly the result of Plk3 activity. This notion is consistent with the observations that Plk3 protein levels remain rather constant during the cell cycle and that Plk3 shares extensive structural homology with Plk1 (24). Therefore, during interphase, Plk3, rather than Plk1, may play a major role in the regulation of MTOC, including its structural integrity and maturation of duplicated centrosomes. The present studies suggest that Plk3 may also have a role during mitosis because it is associated with the mitotic apparatus (Fig. 3). In addition, cells expressing Plk3-A are unable to complete cytokinesis, indicating its role in regulating mitotic exit. These observations are also consistent with early reports that Polo and CDC5 are required for completion of cytokinesis as well as for mitotic progression (29). However, compared to CDC5 (29), the interference of cytokinesis by Plk3-A does not appear to rely on the Polo domain, because Plk3-A lacks only a short sequence at the amino terminus (24). Clearly, it is necessary to study the subcellular localization of Plk1 and Plk3 in the same cell system in order to understand the respective roles of these two homologues in cell cycle regulation.

Disruption of Plk3 function by enforced expression of Plk3^{K52R} apparently causes disorganization of microtubule structures. This is manifested by the formation of long cytoplasmic extensions or branches (Fig. 5D), reminiscent of neuronal cell differentiation. In fact, an early study has shown that Plk3 expression level is high in brain tissues and that it is involved in the maintenance of long-term synaptic plasticity (14). We propose that Plk3 may regulate microtubule dynamics by upregulation of the minus end activity, resulting in accelerated shortening of microtubules because of its close association with MTOC. Disruption (inhibition) of Plk3 activity upon Plk3^{K52R} expression would thus lead to stabilization of microtubules. This notion is supported by the observation that expression of Plk3-A results in rapid cell shrinkage (Fig. 4 and 5) accompanied by cell cycle arrest (Fig. 7). Moreover, Plk3^{K52R}-expressing cells continue to form cell surface protrusions and granules even in the presence of taxol (Fig. 6B).

Although less pronouced than that of Plk3-A, Plk3^{K52R} also induces apparent G_2 and/or mitotic arrest followed by apoptosis (Fig. 7 and 8). One explanation is that Plk3^{K52R} retains a weak kinase activity (24). Alternatively, the cell cycle arrest and apoptosis induced by Plk3^{K52R} and Plk3-A may be mediated by different mechanisms. The latter scenario is supported by the observations that Plk3-A arrests cells at the M/G1 junction, whereas Plk3^{K52R} primarily induces cytoplasmic membrane extensions followed by membrane shedding or fragmentation. Moreover, whereas Plk3^{K52R} expression induced a persistent increase in cyclin B levels (Fig. 7B, lanes 3, 5, and 7), Plk3-A-induced increase in cyclin B expression was short lived (Fig. 7B, lanes 6 and 8), consistent with the notion that cells expressing Plk3-A are G_1-like before undergoing apoptosis. Further studies are needed to determine the underlying mechanisms of these mutants on cell cycle status.

Recent studies have indicated that the phosphorylation of microtubule-associated proteins plays a critical role in the regulation of microtubule stability (2, 5). Several *Drosophila* pro-

teins, such as DMAP-85 (*Drosophila* 85-kDa microtubule-associated protein) and Asp (abnormal spindle), interact with microtubules and are phosphorylated in vitro by Polo (2, 5). Given that both Asp and DMAP-85 associate also with centrosomes or microtubules and that their interactions are regulated by phosphorylation (2, 5) it is tempting to speculate that these human homologues are potential in vivo targets of Plk3.

Centrosome abnormalities are implicated in chromosomal instability and in the development of cancer, and in fact, many cancer cells display multiple centrosomes or enhanced centrosomal activity (20). We have previously shown that Plk3 phosphorylates the oncogene product Cdc25C on serine-216 (23), a site phosphorylation of which should result in downregulation of its activity. Recently, we have also demonstrated that Plk3 phosphorylates p53 on serine-20, an activating phosphorylation site of the tumor suppressor protein (32). Given that Plk3 is downregulated in several types of cancer (3, 18), it is possible that deregulated centrosome or MTOC activities as a result of Plk3 deficiency may contribute to the development and/or progression of these cancers.

ACKNOWLEDGMENTS

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Plk3 Functionally Links DNA Damage to Cell Cycle Arrest and Apoptosis at Least in Part via the p53 Pathway*

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Polo-like kinase 3 (Plk3, previously termed Prk) contributes to regulation of M phase of the cell cycle (Ouyang, B., Pan, H., Lu, L., Li, J., Stambrook, P., Li, B., and Dai, W. (1997) *J. Biol. Chem.* 272, 28646–28651). Plk3 physically interacts with Cdc25C and phosphorylates this protein phosphatase predominantly on serine 216 (Ouyang, B., Li, W., Pan, H., Meadows, J., Hoffmann, I., and Dai, W. (1999) *Oncogene* 18, 6029–6036), suggesting that the role of Plk3 in mitosis is mediated, at least in part, through direct regulation of Cdc25C. Here we show that ectopic expression of a kinase-active Plk3 (Plk3-A) induced apoptosis. In response to DNA damage, the kinase activity of Plk3 was rapidly increased in an ATM-dependent manner, whereas that of Plk1 was markedly inhibited. Recombinant Plk3 phosphorylated *in vitro* a glutathione *S*-transferase fusion protein containing p53, but not glutathione *S*-transferase alone. Recombinant Plk1 also phosphorylated p53 but on residues that differed from those targeted by Plk3. Co-immunoprecipitation and pull-down assays demonstrated that Plk3 physically interacted with p53 and that this interaction was enhanced upon DNA damage. *In vitro* kinase assays followed by immunoblotting showed that serine 20 of p53 was a target of Plk3. Furthermore, expression of a kinase-defective Plk3 mutant ($Plk3^{K52R}$) resulted in significant reduction of p53 phosphorylation on serine 20, which was correlated with a decrease in the expression of p21 and with a concomitant increase in cell proliferation. These results strongly suggest that Plk3 functionally links DNA damage to cell cycle arrest and apoptosis via the p53 pathway.

In mammals, DNA damage induced by ionizing radiation or UV light is detected by two serine/threonine kinases known as "mutated in ataxia telangiectasia" (ATM)[1] and "ATM-related" (ATR) (3–5). Depending on the extent of DNA damage, cells either undergo cell cycle arrest or initiate apoptosis, responses that are at least partly mediated by p53 (4–6). Activated ATM and ATR phosphorylate p53 on serine 15 (7–9), thereby contributing to the activation of the tumor suppressor protein. The kinases Chk1 and Chk2, which act downstream of ATR and ATM, respectively (10–12), are reported to phosphorylate p53 *in vitro* on serine 20 (13–14); this residue is located within the domain of the protein that interacts with HDM2, resulting in stabilization of the normally short lived p53 protein in response to DNA damage (15).

Polo family kinases also play a role in the DNA damage response (11, 16, 18). Cdc5, a polo homolog in budding yeast, is modified in its mobility on denaturing gels in response to DNA damage, and this modification is dependent on MEC1, Rad53 (a Chk homolog), and Rad9 (16). In addition, a functionally defective Cdc5 mutant suppresses a Rad53 checkpoint defect, whereas overexpression of Cdc5 overrides checkpoint-induced cell cycle arrest (11). Moreover, DNA damage appears to interfere with the activation of Plk1 in mammals, resulting in down-regulation of the kinase activity of this protein (18). On the other hand, expression of Plk1 mutants that are nonresponsive to DNA damage overrides G_2 arrest. Mammalian Plk3 is a structural homolog of Plk1 (19), and its expression is down-regulated in several types of cancer (19, 20). Both Plk1 and Plk3 can rescue the temperature-sensitive phenotype of yeast Cdc5 mutants (1, 21). However, evidence indicates that Plk3 functions differently from Plk1 in regulation of cell proliferation and oncogenesis in mammalian cells (1, 2, 20).

We have previously shown that Plk3 phosphorylates Cdc25C on serine 216 (2), a site that is also targeted by Chk1 and Chk2 (10, 22). Phosphorylation of serine 216 of Cdc25C is inhibitory, which is due to sequestration of the protein phosphatase in the cytoplasm by 14-3-3 protein (24). In this report, we have provided evidence indicating that Plk3 is involved in DNA damage checkpoint response and that it may target p53 *in vivo* through regulation of phosphorylation on serine 20. A model is proposed that explains the mechanism of action of Plk3 during genotoxic stress-induced activation of the DNA damage checkpoint, which results in cell cycle arrest and/or apoptosis.

EXPERIMENTAL PROCEDURES

Immunoblotting and Pull-down Assays—Various cell lines were obtained from ATCC, except for the GM00637 cell line, which was originally from the Coriell Institute for Medical Research. Cells treated with adriamycin (100 μM) for 30 min were collected and lysed (1). Equal amounts (50 μg) of protein lysates from various cell lines were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies to human Plk3 (Pharmingen) (2, 25), α-tubulin (Sigma), and p53 (Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and Pharmingen). Recombinant His_6-Plk3 expressed with the use of a baculoviral expression system as described (2) was affinity-purified with and subsequently conjugated to Ni^{2+}-NTA resin (Qiagen). Plk3-conjugated Ni^{2+}-NTA resin or the resin alone was incubated for 3 h at 4 °C with lysates (1 mg of protein) of GM00637 cells that had been pretreated for 30 min with adriamycin. After washing of the resin, bound proteins were eluted and subjected to SDS-polyacrylamide gel electrophoresis and immunoblot analysis with antibodies to p53 or serine 20-phospho-

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[1] The abbreviations used are: ATM, mutated in ataxia telagiectasia; ATR, ATM-related; GST, glutathione *S*-transferase; NTA, nitrilotriacetic acid.

rylated p53 (New England Biolabs). The p53 antigens were detected with horseradish peroxidase-conjugated goat secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech). Each experiment was repeated at least three times.

Co-immunoprecipitation Analysis—GM00637 cell lysates (1 mg of protein) were incubated for 30 min at room temperature in a total volume of 500 μl with of 20 μl of protein A/G-agarose bead slurry (Santa Cruz Biotechnology). After removal of the beads, the supernatant was supplemented with either rabbit polyclonal (Pharmingen) or mouse monoclonal antibodies to p53 or with mouse monoclonal antibodies to CD45 (Pharmingen), followed by incubation for an additional 2 h at room temperature or overnight at 4 °C. Protein A/G-agarose beads (20 μl) were then added to each immunoprecipitation mixture, and the incubation was continued for 1 h at room temperature. Immunoprecipitates were collected by centrifugation, washed three times with the cell lysis buffer, and subjected to immunoblot analysis with a monoclonal antibody to Plk3.

Immunocomplex Kinase Assays—Immunocomplex kinase assays were performed essentially as described (1). In brief, A549 or ATM-deficient (ATCC number CRL-7201) (23) cells were exposed to adriamycin for various times, lysed, and subjected to immunoprecipitation with antibodies to Plk3 or to Plk1 (Zymed Laboratories Inc.). The resulting precipitates were resuspended in kinase buffer (10 mM Hepes-NaOH (pH 7.4), 10 μM $MnCl_2$, 5 mM $MgCl_2$), and the kinase reaction was initiated by the addition of [γ-^{32}P]ATP (2 μCi) (Amersham Pharmacia Biotech) and either α-casein (Sigma), GST-p53, or GST. After incubation for 30 min at 37 °C, the reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The phosphorylated α-casein was quantified by densitometric scanning. Recombinant His_6-Plk3, His_6-Plk1, and His_6-Plk3^{K52R} were also assayed for kinase activity as controls. In some kinase assays, GST-p53 or GST-p53^{S20A} was incubated with His_6-Plk3 in the kinase buffer supplemented with "cold" ATP. After reaction, GST-p53 and GST-p53^{S20A} were blotted for serine 20 phosphorylation. Each assay was repeated for at least three times.

Phosphopeptide Mapping—Tryptic peptide mapping was performed essentially as described (2, 26). In brief, ^{32}P-labeled p53 was excised from dried SDS-polyacrylamide gels and eluted into extraction buffer (50 mM NH_4HCO_3, 0.1% SDS, 1% 2-mercaptoethanol). After removal of debris by centrifugation, the eluted protein was supplemented with 100 μg of acetylated bovine serum albumin (Sigma) and then precipitated by the addition of trichloroacetic acid. The protein precipitate was recovered by centrifugation, washed once with ethanol, dissolved in performic acid (98% formic acid, 30% H_2O_2; 9:1 (v/v)), and lyophilized. The dried protein was resuspended in 50 mM NH_4HCO_3 (pH 8.0) and subjected to digestion with L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (Sigma). The resulting peptides were applied to thin layer chromatography plates and fractionated by electrophoresis followed by chromatography as described (26) with a Multiphor II (Amersham Pharmacia Biotech) apparatus. The plates were then air-dried and subjected to autoradiography. The mapping experiment was repeated at least three times.

Transient Transfection—HeLa cells were transfected, using the LipofectAMINE method (Life Technologies, Inc.), with pCR259Plk3^{K52R} (1, 20), pcDNA3-p53, pcDNA3-p53^{S20A}, or the empty vectors (Invitrogen). The serine 20 mutant (serine 20 was replaced with alanine) of p53 was obtained via site-directed mutagenesis using a kit purchased from Stratagene according to the protocol provided by the supplier. One day after transfection, cells were treated with or without 100 μM adriamycin for 1 h. Cell lysates were prepared and blotted for Plk3, p53, or p21 expression. Two different antibodies to p53 were used. One was from Santa Cruz Biotechnology (DO-1, the recognition epitope of which was between residues 11 and 25), and the second one was from Pharmingen (G59-12). To determine the effect of p53 or Plk3^{K52R} on overall cell growth, HeLa cells transfected with p53, p53^{S20A}, and/or Plk3^{K52R} were cultured in medium containing G418 (600 μg/ml) and maintained for 2 weeks. Colonies formed were visualized after staining with 0.125% crystal violet solution. As an alternative method to determine cell proliferation, triplicate transfected cells that were maintained in G418-containing medium (600 μg/ml) for 1 week were subject to 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction assay as described (27). ODs at 570 nm were determined for each sample. Each transfection experiment was repeated at least three times.

DNA Fragment End Labeling Assay—End labeling of DNA fragments was performed using a kit purchased from Oncogene Research Products (Boston, MA) according to the protocol provided by the manufacturer. Briefly, GM00637 cells cultured on cover slides were transfected with Plk3-A expression construct or with the vector alone for 18 h. The transfected cells as well as the untransfected parental cells were washed once with phosphate-buffered saline and then sequentially fixed in 4% paraformaldehyde and 80% ethanol for 10 min each. The fixed cells were rehydrated in Tris-buffered saline for 10 min and then treated with proteinase K (2 mg/ml) for 4 min. The treated cells were incubated in the labeling reaction mixture containing terminal deoxynucleotidyl transferase at 37 °C in a humid chamber for 90 min. After reaction, cells were stained with 4′,6-diamidino-2-phenylindole (1 mg/ml) for 5 min. Fluorescence microscopy was performed on an Olympus AT70 microscope (Spot Diagnostic Instrument), and images were captured using a digital camera using Image System Spot RT software. GM00637 cells transfected with Plk3-A, Plk3^{K52R}, or the vector alone for 1 day were also collected for genomic DNA isolation. The isolated DNA (10 μg/lane) was analyzed on agarose gels.

RESULTS

Plk3 is a structurally conserved protein serine/threonine kinase playing an important role in the regulation of M phase function (1). A recent study indicates that overexpression of Plk3 induces chromatin condensation and programmed cell death (25). To confirm the role of Plk3 in induction of apoptosis, we transfected GM00637 fibroblast cells with a plasmid construct expressing kinase-active Plk3 (Plk3-A) (1, 2). DNA fragmentation analyses (Fig. 1) revealed that transfection of Plk3-A, but not the vector alone, induced significant DNA fragmentation, indicative of apoptosis. Transfection of Plk3^{K52R} in which lysine 52 was replaced with arginine also induced DNA fragmentation, albeit it was less significant than that of Plk3-A (Fig. 1*B*).

Recent studies have shown that phosphorylation of Cdc25C is inhibitory (22, 24). The observations that Plk3 phosphorylates serine 216 of Cdc25C (2) and that polo family kinases contribute to regulation of the DNA damage checkpoint (11, 16, 18) prompted us to investigate whether the kinase activity of Plk3 is affected in cells subjected to DNA damage. Immunocomplex kinase assays with α-casein as substrate revealed that the kinase activity of Plk3 was increased more than 10-fold by exposure of A549 cells to the DNA-damaging agent adriamycin (Fig. 2, *A* and *B*). A549 cells were used because they expressed good levels of Plk3. The activation of Plk3 in response to other genotoxic stresses such as UV or H_2O_2 was also detected (data not shown). Immunoblot analysis indicated (Fig. 2*A*) that Plk3 antigen was not increased upon adriamycin treatment, suggesting that the increase of Plk3 kinase activity was due to a post-translational mechanism(s). Given that the kinase activity of Plk1 has been shown to be down-regulated during activation of the DNA damage checkpoint (18) and that the antibody to Plk3 used for our immunocomplex kinase assay did not cross-react with human Plk1 (Fig. 2*C*), we measured the kinase activities of both Plk1 and Plk3 in the same A549 cells treated with adriamycin. Whereas little endogenous Plk3 kinase activity was detected under control conditions (Fig. 2*D*, *lane 1*), activation of Plk3 was apparent 10 min after exposure of the cells to adriamycin; in contrast, Plk1 was constitutively active under basal conditions (Fig. 2*D*, *lane 4*), and its activity was markedly inhibited in response to adriamycin treatment (*lanes 5* and *6*). Thus, Plk1 and Plk3 appear to be differentially regulated in response to DNA damage.

To examine whether the DNA damage-induced activation of Plk3 is dependent on ATM, we exposed A549 cells that had been pretreated with caffeine, which inhibits the kinase activities of ATM and ATR (28), to adriamycin. Caffeine not only blocked the activation of Plk3 by adriamycin but also inhibited the basal kinase activity of this protein (Fig. 3, *A* and *B*). To further confirm the dependence of Plk3 activation on ATM, we analyzed the Plk3 kinase activity in an ATM-deficient cell line (ATCC number CRL-7201) that had been treated with adriamycin for various times. No increase in Plk3 activity upon DNA damage was detected in the ATM-deficient cells (Fig. 3, *C* and *D*, *lanes 2–4*).

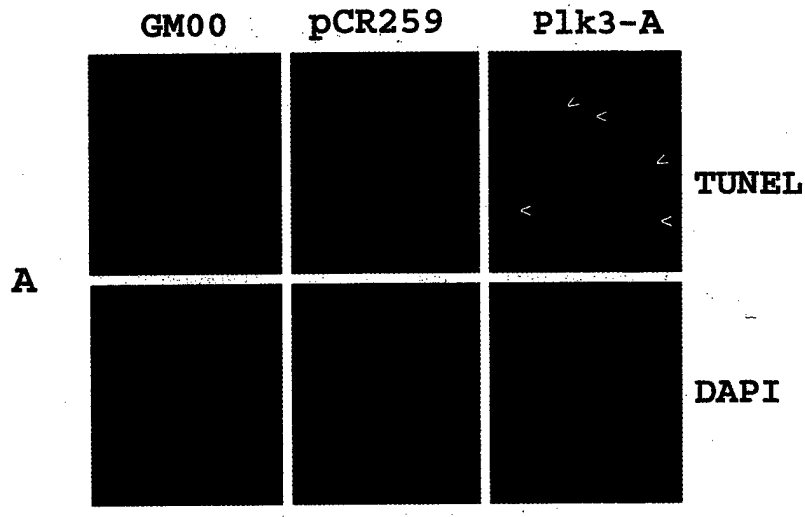
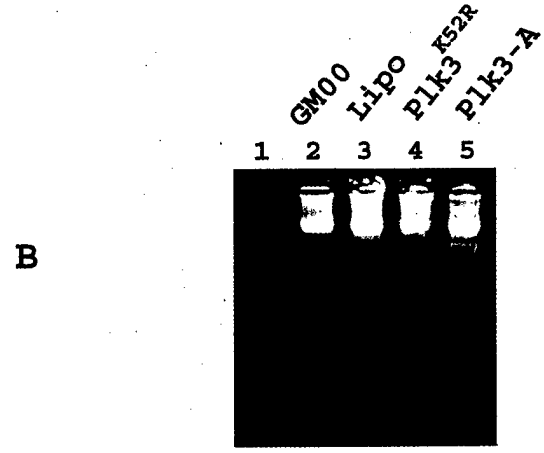


FIG. 1. **Ectopic expression of Plk3 induces apoptosis.** GM00637 cells transfected with the Plk3-A expression construct or the vector were analyzed for DNA fragmentation using a DNA fragment end labeling (FragEL™) detection kit as described under "Experimental Procedures" (*A*) or via agarose gel electrophoresis (*B*). Untransfected parental cells were also used for analyses. Representative results from three similar experiments were presented.



In addition to their reported phosphorylation of p53 on serine 20 (14, 29, 30), Chk1 and Chk2 phosphorylate serine 216 of Cdc25C (11, 22), phosphorylation of which is thought to be partly responsible for G_2 arrest during activation of the DNA damage checkpoint. Given that Plk3 phosphorylates the same residue of Cdc25C (serine 216) as that targeted by Chk1 and Chk2, we investigated whether Plk3 also phosphorylates p53 *in vitro*. Recombinant histidine-tagged Plk3-A (His_6-Plk3-A) phosphorylated GST-p53 (Fig. 4*A*, *lane 2*) but not GST alone (*lane 5*), indicating that Plk3 targets the p53 moiety of GST-p53. $Plk3^{K52R}$ phosphorylated GST-p53 to a greatly reduced extent (Fig. 4*A*, *lane 3*) compared with that observed with Plk3-A (*lane 2*). Recombinant (His_6)-Plk1 also phosphorylated GST-p53 (Fig. 4*A*, *lane 9*) but not GST alone (data not shown). Unlike His_6-Plk3, His_6-Plk1 exhibited a high level of autophosphorylation activity (Fig. 4*A*, *lane 9*).

The biological functions of mammalian Plk3 and Plk1 appear to differ (2, 19, 20, 32, 33), although both mammalian proteins complement Cdc5 temperature-sensitive mutants of budding yeast (1, 21). Overexpression of murine Plk1 results in oncogenic transformation (33), whereas ectopic expression of human Plk3 inhibits cell growth by inducing apoptosis (25). Thus, our observation that both Plk3 and Plk1 phosphorylate p53 suggested that these two kinases may target different residues of the tumor suppressor protein. Whereas p53 phosphorylated by Plk3 yielded two major phosphopeptides in phosphopeptide mapping analysis, p53 phosphorylated by Plk1 yielded four major phosphopeptides (Fig. 4*B*). Further analysis of the phosphopeptide maps indicated that peptides a and e were specific to Plk3-phosphorylated p53 and that peptides b, c, and d were unique to Plk1-phosphorylated p53.

Our observation that Plk3 phosphorylates p53 *in vitro* suggested that the two proteins might physically interact *in vivo*. Co-immunoprecipitation experiments (Fig. 5*A*) revealed that both antibodies (from two different sources) to p53, but not control antibodies to CD45, precipitated Plk3 from GM00637 cell lysates. Furthermore, Ni^{2+}-NTA resin conjugated with His_6-Plk3, but not Ni^{2+}-NTA resin alone, precipitated p53 from the cell lysates (Fig. 5*B*). The amount of p53 precipitated by the His_6-Plk3-conjugated resin was markedly increased by prior exposure of the cells to adriamycin (Fig. 5*B*, *lanes 3* and *4*), although treatment with this drug for 30 min did not affect the total amount of p53 present in the cells (*lanes 5* and *6*). Proteins eluted from both His_6-Plk3-conjugated resin and control resin were also blotted for the presence of phospho-p53. Fig. 5*B* shows that His_6-Plk3 resin precipitated serine 20-phosphorylated p53 and that the amount of the phosphorylated p53 pulled down by His_6-Plk3 was significantly increased following adriamycin treatment. These results thus suggest that activation of p53 promotes its interaction with Plk3.

Chk1 and Chk2, as well as Plk3, phosphorylate Cdc25C in serine 216 (2, 10, 22). The direct interaction of Plk3 with p53 and the observation that Chk1 and Chk2 also phosphorylate p53 on serine 20 prompted us to examine whether the serine 20 residue of p53 was a phosphorylation target of Plk3. *In vitro* kinase assays followed by immunoblotting showed (Fig. 5*C*, *lane 2*) that purified GST-p53 was not recognized by an antibody to serine 20-phosphorylated p53. However, GST-p53 phos-

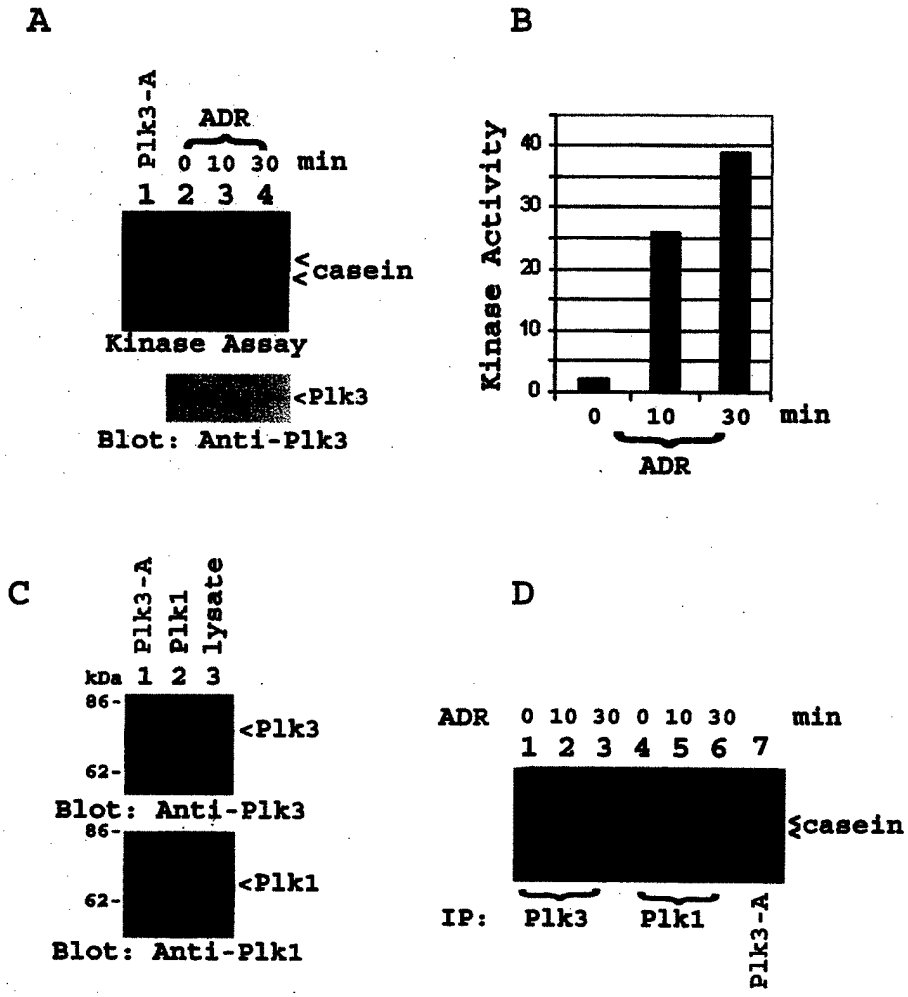


FIG. 2. **Activation of Plk3 in response to DNA damage.** *A*, A549 cells were incubated with 100 μM adriamycin (*ADR*) for the indicated times, after which cells were lysed and subjected to immunoprecipitation (*IP*) with antibodies to Plk3. The resulting precipitates were then assayed for kinase activity with α-casein as substrate. Recombinant His_6-Plk3-A (Plk3-A) was used as a positive control for *in vitro* assay of kinase activity. The *arrowheads* indicate phosphorylated α-casein. The same A549 cell lysate samples were also blotted with antibody to Plk3. *B*, quantification of Plk3 kinase activity as shown in *A*. The kinase units are arbitrary. *C*, duplicate protein blots with purified recombinant His_6-Plk3-A and Plk1, as well as A549 cell lysates, were probed with antibodies to Plk3 and Plk1, respectively. Recombinant Plk1 migrates slightly slower than the cellular one due to addition of a FLAG tag at the amino terminus. *D*, A549 cells were incubated with 100 μM adriamycin for the indicated times, after which cells were lysed and subjected to IP with antibodies to either Plk3 or Plk1, as indicated. The resulting precipitates were assayed for kinase activity. His_6-Plk3-A (*lane 7*) was used as a control for the kinase assay. Presented results were representative of at least three similar experiments.

phorylated *in vitro* by Plk3 exhibited a strong phosphoserine 20 epitope (*lane 3*). Furthermore, when an equal amount of GST-$p53^{S20A}$, a mutant in which serine 20 was replaced with alanine, was used as an *in vitro* substrate, no phosphoserine 20 epitope was detected (Fig. 5*C*, *lane 4*). Given that p53 serine 20 phosphorylation and Plk3 activation are induced by a variety of genome toxic stresses, these observations suggest that the serine 20 residue may be a direct *in vivo* target of Plk3.

To establish Plk3 serine 20 phosphorylation of p53 *in vivo*, we transfected both p53 and $p53^{S20A}$ expression plasmids into HeLa cells, which constitutively express Plk3 but not p53. Western blot analysis showed (Fig. 6*A*) that transfected p53, but not $p53^{S20A}$ mutant protein, was constitutively phosphorylated on serine 20. DNA damage induced by adriamycin significantly enhanced serine 20 phosphorylation of the transfected p53, indicating that the pathway leading to p53 serine 20 phosphorylation was intact, albeit endogenous p53 was inactivated in this tumor cell line.

To determine the effect of change in Plk3 activity on p53 phosphorylation, HeLa cells were cotransfected with plasmid constructs expressing p53 and $Plk3^{K52R}$. Western blot analysis showed (Fig. 6*B*) that p53 was expressed and phosphorylated on serine 20 and that expression of $Plk3^{K52R}$ significantly reduced the level of p53 phosphorylation on serine 20 (*lane 6*). To determine the consequence of serine 20 phosphorylation of p53 on its target gene expression, we measured p21 level in cells expressing p53 and/or the Plk3 dominant mutant. We have consistently observed (Fig. 6*B*) that while little p21 was detected in parental HeLa cells or cells transfected with vectors alone (*lanes 1* and *2*), ectopic expression of p53 greatly induced p21 expression (*lane 3*). Expression of $p53^{S20A}$ also induced p21 expression that was about 25% of that induced by wild-type p53 (*lane 4*). Further, expression of the $Plk3^{K52R}$ greatly reduced the ability of p53 to induce p21 expression (compare *lanes 3* and *6*), which was consistent with the reduced serine 20 phosphorylation of p53 as well as p53 protein levels. These observations thus clearly demonstrate that phosphorylation of p53 on serine 20 plays a significant role in activating p21 expression.

We then determined the colony-forming efficiency of HeLa cells that were cotransfected with $Plk3^{K52R}$ and p53. Fig. 7*A* shows that p53 suppressed colony formation of HeLa cells. $Plk3^{K52R}$ alone also suppressed the colony formation. However, when cotransfected with p53, $Plk3^{K52R}$ significantly blocked p53-mediated suppression of colony formation of HeLa cells. Independent assays using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method confirmed that $Plk3^{K52R}$ effectively blocked the ability of p53 to inhibit HeLa cell proliferation (Fig. 7*B*). It is interesting that $Plk3^{K52R}$ also had an effect on $p53^{S20A}$ in terms of cell growth. This effect was likely to be mediated by inhibition of phosphorylation of other serine/threonine sites by $Plk3^{K52R}$ because Plk3 phosphorylates p53 on multiple sites *in vitro* (Fig. 4*B*).

DISCUSSION

Our results demonstrate that Plk3 contributes to DNA damage checkpoint activation, which is at least partly mediated by regulating phosphorylation of p53. The adriamycin-induced activation of Plk3 was caffeine-sensitive and not observed in

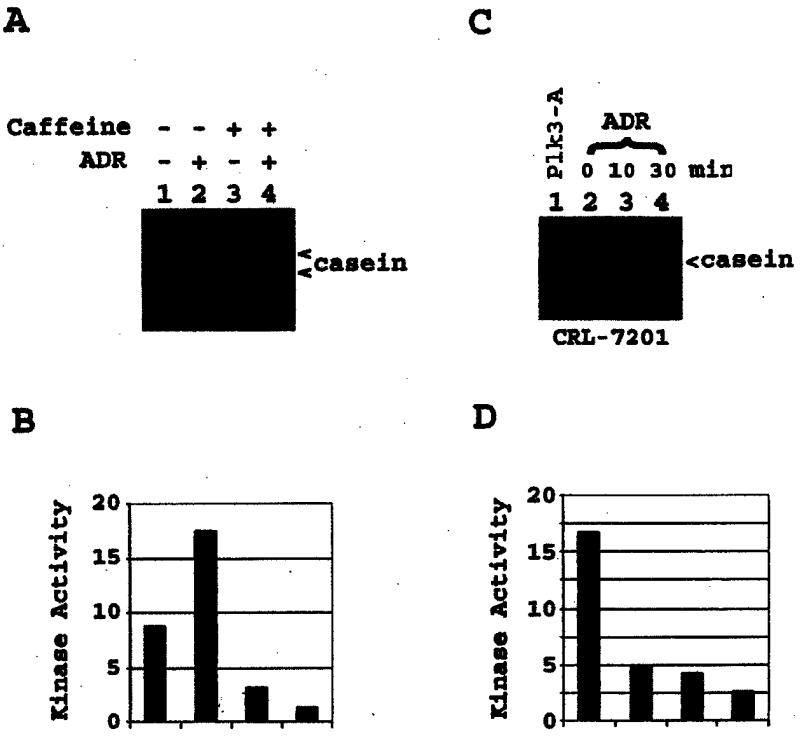


FIG. 3. **Plk3 activation is ATM-dependent.** *A*, A549 cells were preincubated with vehicle (medium) or 5 μM caffeine for 2 h and then incubated with 100 μM adriamycin for 30 min. Plk3 immunoprecipitates were then prepared and assayed for kinase activity. *B*, quantification of Plk3 kinase activity as shown in *A*. Units are arbitrary. *C*, an ATM-deficient cell line CRL-7201 was treated with 100 μM adriamycin for the indicated times, and Plk3 proteins immunoprecipitated from the treated cells were assayed for kinase activity. His6-Plk3-A was used as a control (*lane 1*). *D*, quantification of Plk3 kinase activity as shown in *C*. Units are arbitrary.

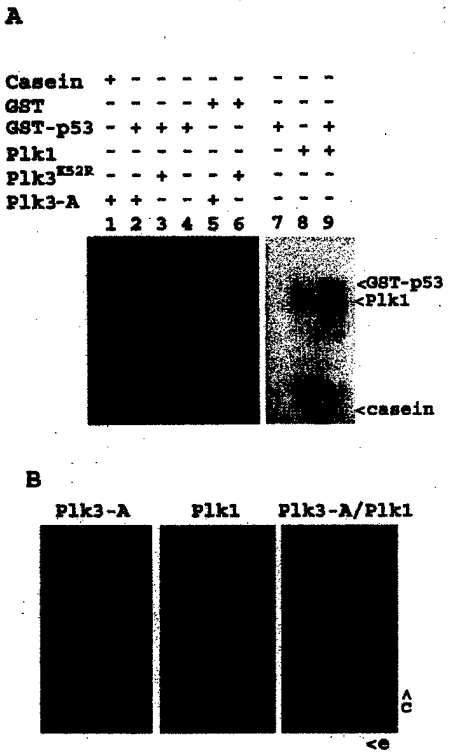


FIG. 4. **Phosphorylation of p53 on different residues by Plk3-A and Plk1.** *A*, recombinant His_6-Plk3-A, His_6-$Plk3^{K52R}$, and Plk1 were assayed for kinase activity with GST-p53 as substrate. α-casein and GST were used as positive and negative control substrates, respectively. *B*, two-dimensional analysis of ^{32}P-labeled tryptic peptides derived from GST-p53 phosphorylated *in vitro* by either His_6-Plk3-A (*left panel*) or His_6-Plk1 (*middle panel*), or mixed samples (*right panel*). The directions of electrophoresis (*e*) and chromatography (*c*) are indicated, as is the origin (*o*).

ATM-deficient cells, indicating the participation of ATM. Phosphorylation is thought to play an important role in regulation of the stability and activity of p53. This study suggests that serine 20 of p53 may be an *in vivo* target of Plk3 during DNA damage checkpoint activation because recombinant Plk3 phosphorylates p53 *in vitro*, resulting in a strong phosphoserine 20 epitope (Fig. 5*C*) and because it also interacts with serine 20-phosphorylated p53. Furthermore, expression of $Plk3^{K52R}$ in HeLa cells results in decreased phosphorylation of cotransfected p53 on serine 20, which was correlated with a significant reduction in p21 expression (Fig. 6*B*) with a concomitant increase in cell proliferation (Fig. 7).

Together with previous observations (2), our data suggest a simple and somewhat redundant set of mechanisms for DNA damage-induced signal transduction between ATM or ATR and effector molecules, resulting in cell cycle arrest and apoptosis (Fig. 8). According to this model, Plk3 may act in parallel with Chk1 and Chk2, downstream of ATM or ATR. It is possible that Plk3 preferentially transduces signals generated by a specific genotoxic stress, just as Chk1 and Chk2 are differentially activated by UV radiation and ionizing radiation, respectively (6). Alternatively, Plk3 may be activated by Chk2 (and/or Chk1), given that Cdc5 acts downstream of Rad53 in yeast (16). Plk3 may thus integrate the signals from ATM-Chk2 and ATR-Chk1 and induce cell cycle arrest or apoptosis by phosphorylating both p53 and Cdc25C. Consistent with this second scenario, Plk3 is activated by the ionizing radiation-mimetic drug adriamycin, UV radiation, and oxidative stress.[2]

A third possibility also exists to explain our observations; namely Chk1 and Chk2 preferentially target serine 20 of p53. In this case, p53, phosphorylated on serine 20 by Chk1 and Chk2, interacts with high affinity to Plk3, resulting in phosphorylation of p53 on additional sites. This would also place Plk3 downstream of Chk1/Chk2 in the DNA damage checkpoint pathway. It is possible that phosphorylation of p53 on other sites enforces the DNA damage checkpoint, but is not absolutely required. Although we observed that $Plk3^{K52R}$ expression reduced levels of serine 20 phosphorylation of p53, overexpression of this mutant protein may result in more efficient interaction with p53, potentially blocking access of p53 to

[2] S. Xie, H. Wu, Q. Wang, and W. Dai, unpublished data.

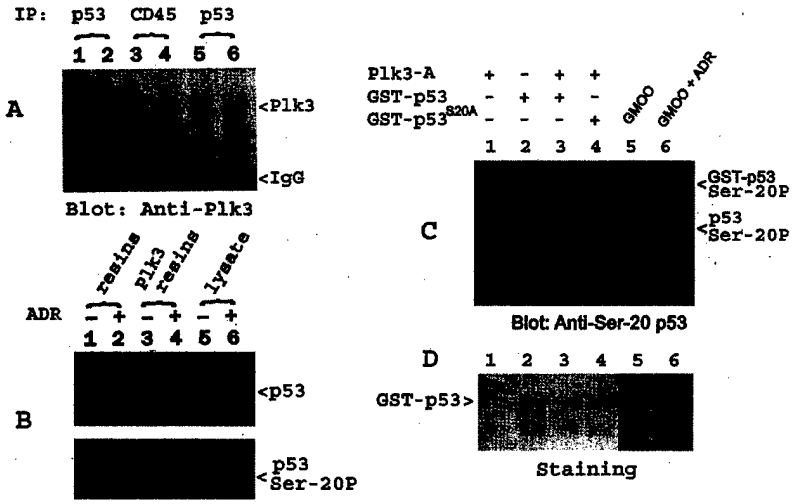
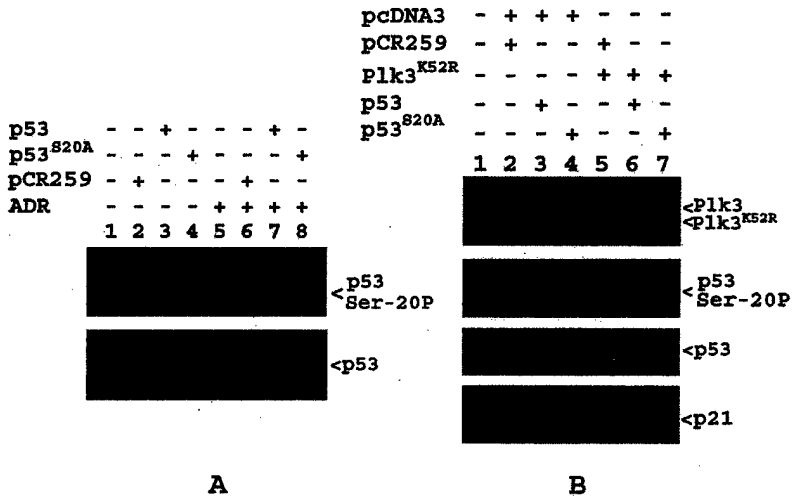


FIG. 5. **Plk3 interacts with p53 and phosphorylates the tumor suppressor protein on serine 20.** *A*, lysates from GM00637 cells were subjected to immunoprecipitation (*IP*) with polyclonal (*lanes 1* and *2*) or monoclonal (*lanes 5* and *6*) antibodies to p53 or with monoclonal antibodies to CD45 as a negative control (*lanes 3* and *4*). The positions of bands corresponding to Plk3 and immunoglobulin G (IgG) are indicated. *B*, His_6-Plk3-A immobilized on Ni^{2+}-NTA resin (*lanes 3* and *4*) or resin alone (*lanes 1* and *2*) was incubated with lysates of GM00637 cells that had been pretreated for 30 min with (*lanes 2* and *4*) or without (*lanes 1* and *3*) adriamycin. Proteins that interacted specifically with the His_6-Plk3-A-conjugated resin or resin alone were eluted and subjected to immunoblot analysis with antibodies to p53 or serine 20-phosphorylated p53. 50 μg of the cell lysates were also directly subjected to immunoblot analysis (*lanes 5* and *6*). *C*, GST-p53 and GST-$p53^{S20A}$ were incubated *in vitro* in the kinase buffer with His_6-Plk3-A, and the phosphorylated proteins (*lanes 3* and *4*) as well as the nonphosphorylated GST-p53 (*lane 2*) were subject to immunoblot analysis with antibodies to serine 20-phosphorylated p53. Lysates from GM00637 cells treated with or without adriamycin were used as controls for immunoblotting. *D*, the blot as shown in *C* was stained with Coomassie Blue as a loading control. Some degradations of GST-p53 were observed. The results are representative of three similar experiments.

FIG. 6. *A*, HeLa cells transfected with p53, $p53^{S20A}$, or vector were treated with or without adriamycin for 30 min. Cell lysates were then analyzed for serine 20-phosphorylated p53 as well as total p53. The antibody to p53 recognized an epitope between residues 11 and 25 (Santa Cruz Biotechnology). Thus, serine 20 mutation significantly compromised the detection. *B*, HeLa cells were cotransfected with various expression plasmids for 24 h as indicated. Cell lysates were collected and blotted for Plk3, serine 20-phosphorylated p53, total p53, and p21. Recognition of total p53 by the p53 antibody (Pharmingen) was not affected by the serine 20 mutation. The results were representative of three independent experiments.



Chk1 or Chk2. Therefore, the *in vivo* role of Plk3 in regulating serine 20 phosphorylation remains to be established, which would require obtaining Plk3 null cells.

Several other residues of p53 are potential sites of phosphorylation by Plk3. Phosphorylation of threonine 18 is induced by DNA damage, and a casein kinase 1-like enzyme is thought to phosphorylate this residue in A549 cells (31). However, the identity of this casein kinase 1-like enzyme is unknown. Given that Plk3, a casein-phosphorylating kinase, is activated by DNA damage, Plk3 may also target threonine 18 of p53. Moreover, given the apoptosis-inducing function of Plk3 (Fig. 1) and the observations both that residues 43–63 of p53 are necessary for induction of apoptosis by p53 (23) and that serine 46 phosphorylation is important for the transactivation of apoptosis-inducing genes by p53 (17), serine 46 may be another potential site of phosphorylation of p53 by Plk3.

Overexpression of Plk3-A induced a significant level of DNA fragmentation in GM00637 cells (Fig. 1), which is consistent with an early finding that Plk3 induces chromatin condensation and apoptosis (25). Given that p53 is an *in vitro* substrate of Plk3-A, it is reasonable to predict that Plk3-induced apoptosis may be mediated through phosphorylation and activation of p53. However, we were unable to detect enhanced serine 20 phosphorylation of p53 in HeLa cells cotransfected with Plk3-A and p53 expression constructs (data not shown). One explanation for this discrepancy is that the Plk3-A clone is missing a short nucleotide sequence encoding about 30 amino acid residues at the amino terminus when compared with that of murine counterpart (19). Although it is active *in vitro*, Plk3-A may be much less so than the cellular Plk3 toward *in vivo* targets. Thus, it is possible that Plk3-A may not behave as a "wild-type" protein *in vivo*, resulting in only a partial response to the upstream activators. Alternatively, HeLa cells may lack a cofactor(s) that is required for activation of Plk3, or the pathway leading activation of Plk3 is compromised.

It is interesting to note that $Plk3^{K52R}$ also suppressed colony

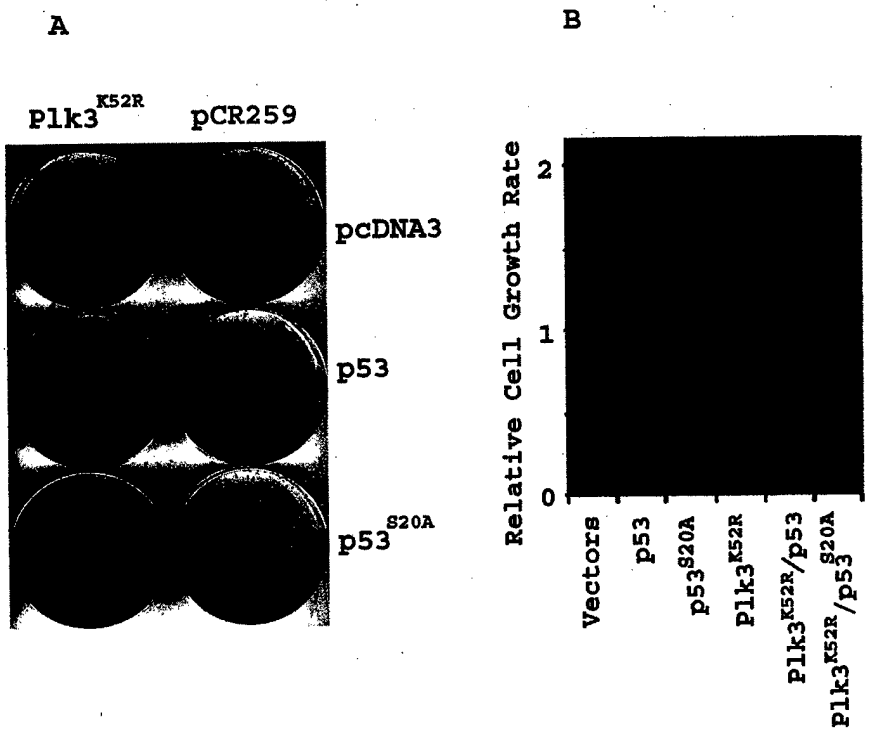


FIG. 7. **Plk3^{K52R} blocks p53-mediated suppression of cell proliferation.** *A*, HeLa cells transfected with p53, p53^{S20A}, and/or Plk3^{K52R} expression constructs were cultured in medium containing G418. Colonies were visualized after staining with crystal violet after 2 weeks' incubation. *B*, HeLa cells transfected with p53, p53^{S20A}, and/or Plk3^{K52R} expression constructs were cultured in the medium containing G418. After 1 week's incubation, cell proliferation rate was measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Relative growth rates (A_{570}) were summarized from three independent experiments.

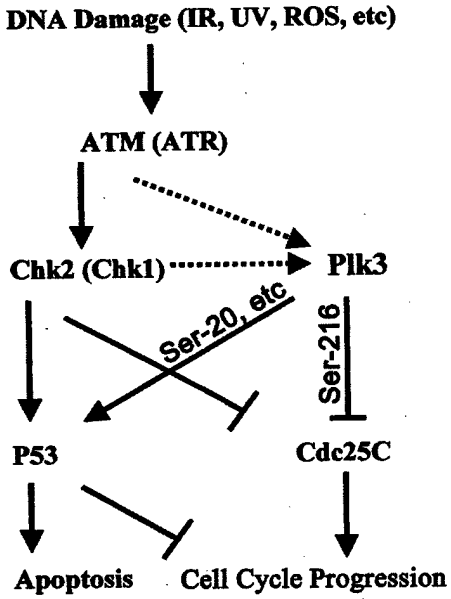


FIG. 8. **A model for the regulation of cell cycle progression by Plk3 in response to DNA damage.** *Dashed arrows* indicate that the direct relationship between these two proteins is not confirmed experimentally.

formation (Fig. 7), which may be partly due to the fact that this mutant protein retains some residual kinase activities toward p53 (Fig. 4*A*) and other *in vitro* substrates such as Cdc25C (2) and α-casein.[2] Given that Plk3-A differs from Plk3^{K52R} by only one amino acid (lysine 52) and that it is more efficient than Plk3^{K52R} in inducing cell cycle arrest and apoptosis (Fig. 1*B*),[2] it is reasonable to conclude that the kinase activity of Plk3 is involved in the regulation of cell proliferation. On the other hand, as shown by the study of Conn *et al.* (25), the COOH-terminal half of Plk3 also plays an important role in induction of apoptosis. In addition, our recent studies indicate that Plk3 is concentrated at the centrosomal region during interphase and that Plk3 appears to be involved in the regulation of microtubule dynamics as well as centrosome function.[3] Therefore, Plk3 may regulate cell proliferation through multiple mechanisms.

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The physical association and phosphorylation of Cdc25C protein phosphatase by Prk

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prk **encodes a protein serine/threonine kinase involved in regulating M phase functions during the cell cycle. We have expressed His6-Prk and His6-Cdc25C proteins using the baculoviral vector expression system. Purified recombinant His6-Prk, but not a kinase-defective mutant His6-Prk^{K52R}, is capable of strongly phosphorylating His6-Cdc25C *in vitro*. Co-immunoprecipitation and affinity column chromatography experiments demonstrate that GST-Prk and native Cdc25C interact. When co-infected with His6-Prk and His6-Cdc25C recombinant baculoviruses, *sf*-9 cells produce His6-Cdc25C antigen with an additional slower mobility band on denaturing polyacrylamide gels compared with cells infected with His6-Cdc25C baculovirus alone. In addition, His6-Cdc25C immunoprecipitated from *sf*-9 cells co-infected with His6-Prk and His6-Cdc25C baculoviruses, but not with His6-Prk^{K52R} and His6-Cdc25C baculoviruses, contains a greatly enhanced kinase activity that phosphorylates His6-Cdc25C *in vitro*. Moreover, phosphopeptide mapping shows that His6-Prk phosphorylates His6-Cdc25C at two sites *in vitro* and that the major phosphorylation site co-migrates with the one that is phosphorylated *in vivo* in asynchonized cells. Further studies reveal that His6-Prk phosphorylates Cdc25C on serine^{216}, a residue also phosphorylated by Chk1 and Chk2. Together, these observations strongly suggest that Prk's role in mitosis is at least partly mediated through direct regulation of Cdc25C.**

Keywords: Cdc25C; Prk; protein kinase; protein phosphatase; cell cycle

Introduction

Cyclin-dependent kinase (CDK) p34^{cdc2} plays an essential role in cell cycle progression, and its kinase activity is under complicated but very tight regulation during the cell cycle. p34^{cdc2} alone is not active as its protein kinase activity is strictly dependent on association with cyclin B, whose level fluctuates during the cell cycle. p34^{cdc2} also undergoes post-translational modification via reversible phosphorylation. For example, CAK (CDK-activating kinase) phosphorylates p34^{cdc2} on threonine 161, activating the kinase, whereas Wee1 kinase phosphorylates p34^{cdc2} on tyrosine 15 (Tyr15) and threonine 14 (thr14), inactivating the kinase (Feilotter *et al.*, 1992; Norbury *et al.*, 1992). On the other hand, the Cdc25C gene product, a dual specific phosphatase, dephosphorylates p34^{cdc2} on Tyr^{15} and Thr^{14} and, thus, positively regulates p34^{cdc2} kinase activity (Feilotter *et al.*, 1992; Norbury *et al.*, 1992).

In human, three structurally related genes exist in the Cdc25C family (Galaktionov and Beach, 1991); they are named Cdc25C A, B, and C. Cdc25A appears to be involved primarily in G1 progression and G1/S transition (Galaktionov *et al.*, 1996). Cdc25B is essential for cell cycle progression, possibly regulating G2/M transition as a 'starter' phosphatase (Lammer *et al.*, 1998). Among the three Cdc25 family genes, Cdc25C is best characterized. Cdc25C is activated in G2, and its activity is critical for the onset and progression of mitosis (Sadhu *et al.*, 1990). In *Xenopus*, Cdc25C is a phosphoprotein and is heavily phosphorylated by kinases other than Cdc2 and Cdk2 during G2 (Izumi and Maller, 1995).

In the past few years, an emerging family of protein kinases (the polo kinase family) has been described in yeast (Kitada *et al.*, 1993), *Drosophila* (Llamazares *et al.*, 1991), *Xenopus* (Kumagai and Dunphy, 1996), mouse (Simmons *et al.*, 1992; Golsteyn *et al.*, 1995; Donohue *et al.*, 1995), and human (Hamanaka *et al.*, 1994; Li *et al.*, 1996; also see recent review Nigg, 1998). The *Drosophila* polo gene, homologous to the budding yeast Cdc5, encodes a serine/threonine (Ser/Thr) kinase and is required for mitosis in this species; mutations in this gene result in abnormal mitotic and meiotic division (Fenton and Glover, 1993). The abnormalities of these mutants are manifested as over-condensed chromosomes, abnormal spindle formation, and polyploidy (Llamazares *et al.*, 1991; Fenton and Glover, 1993). *polo* transcripts are abundantly expressed in tissues and developmental stages that display extensive mitotic activity. The polo kinase activity peaks cyclically at anaphase/telophase (Llamazares *et al.*, 1991; Fenton and Glover, 1993).

The human has at least three genes, *plk* (Hamanaka *et al.*, 1994), *prk* (Li *et al.*, 1996) and *snk* (GenBank accession #AF059617), whose structures are related to *Drosophila*. We have previously reported the cloning and characterization of a cDNA coding for human *prk* (**p**roliferation-related **r**inase or **p**olo-related **k**inase) (Li *et al.*, 1996). Subsequently, we demonstrate that Prk is involved in regulating M phase functions (Ouyang *et al.*, 1997). Our current studies report Prk's mode of action during cell cycle regulation. We show that Prk specifically interacts with and phosphorylates Cdc25C protein phosphatase.

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Results

Our early studies have demonstrated the role of *prk* in the regulation of mitosis and meiosis (Ouyang *et al.*, 1997). Our preliminary study also shows that Prk phosphorylates Cdc25C *in vitro* (Ouyang *et al.*, 1997). To study the mode of action of Prk during cell cycle regulation, we expressed both human His6-Prk and His6-Cdc25C in insect *sf*-9 cells through the baculoviral vector expression system. Figure 1a shows that Prk antigen is expressed in *sf*-9 cells infected with either His6-Prk baculovirus (lane 5) or His6-Prk/His6-Cdc25C baculoviruses (double-infection, lane 3). The same blot was stripped and reprobed with the anti-Cdc25C antibody. Figure 1b shows that Cdc25C antigen is detected in His6-Cdc25C baculovirus-infected cells (lane 4) or the double-infected cells (lane 3). We also expressed His6-tagged Cdc25C in *E. coli* using a prokaryotic expression vector. As shown in Figure 1b, Cdc25C was highly induced after IPTG addition (lane 1) as compared with the non-induced bacterial lysates (lane 2). Figure 1c illustrates the Commassie blue stained blot as a loading control.

We purified recombinant His6-Prk and His6-Cdc25c proteins using affinity chromatography. Purified His6-Prk was analysed for *in vitro* kinase activity using casein or His6-Cdc25C as a substrate. Figure 2a shows that His6-Prk strongly phosphorylated casein (CSN, lanes 1 and 4) but not histone H1 (data not shown) *in vitro*. His6-Prk also phosphorylated the purified His6-Cdc25C *in vitro* (Figure 2a, lanes 3 and 4). To determine whether His6-Prk phosphorylates other members of the Cdc25 family, we expressed and purified GST-Cdc25A and GST-Cdc25B. An equal amount of the purified Cdc25 proteins were used for *in vitro* kinase assays. It was shown (Figure 2b) that His6-Prk phosphorylated all three GST-Cdc25 proteins *in vitro*. It appears that His-Prk phosphorylated Cdc25C (lane 3) more efficiently than it did Cdc25A (lane 1) or Cdc25B (lane 2). To eliminate the possibility that a contaminated protein kinase activity was co-purified with His6-Prk and, was thus actually responsible for the phosphorylation of Cdc25 proteins, we changed a conserved lysine residue (amino acid #52, Ouyang *et al.*, 1997) in the kinase domain of His6-Prk into an arginine residue via site-directed mutagenesis. The mutated molecule His6-Prk^{K52R} was expressed, purified, and assayed for its kinase activity in the same manner as was the wild-type His6-Prk. Figure 2c shows that His6-Prk^{K52R} weakly phosphorylated His6-Cdc25C (lane 4) whereas His6-Prk phosphorylated His6-Cdc25C efficiently (lane 3), indicating that Prk is the primary kinase that phosphorylates the phosphatase. Titration experiments confirmed that Prk^{K52R} had a

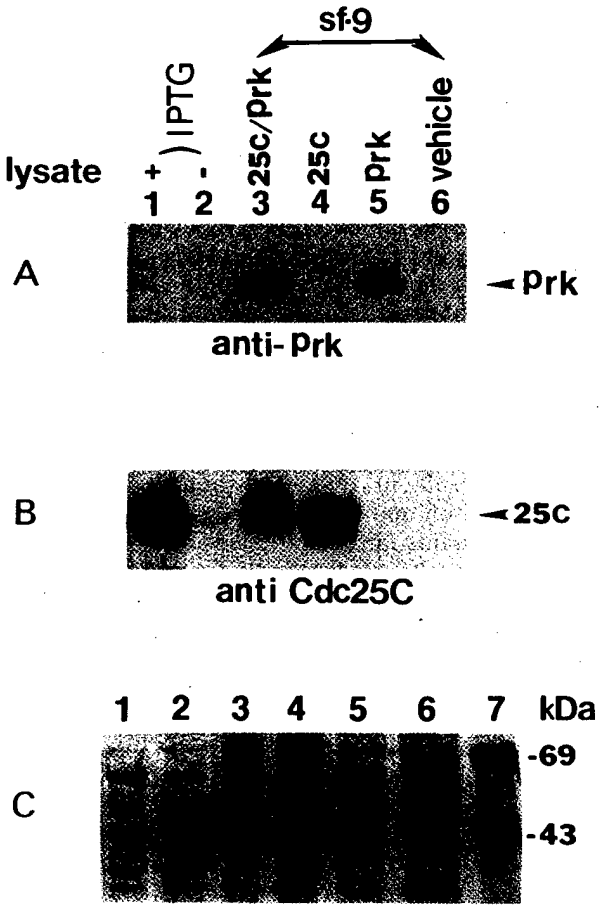

Figure 1 Analysis of human His6-Prk and His6-Cdc25C expression in *sf*-9 cells. *sf*-9 cells infected with the wild-type baculovirus (vehicle), His6-Prk, His6-Cdc25C, or His6-Prk/His6-Cdc25C recombinant baculoviruses were analysed for Prk (**a**) or Cdc25C (**b**) expression through Western blotting. His6-Cdc25C was also expressed using a prokaryotic expression vector in the presence (lane 1) or absence (lane 2) of IPTG. (**c**) Commassie blue staining of the protein blot as a loading control

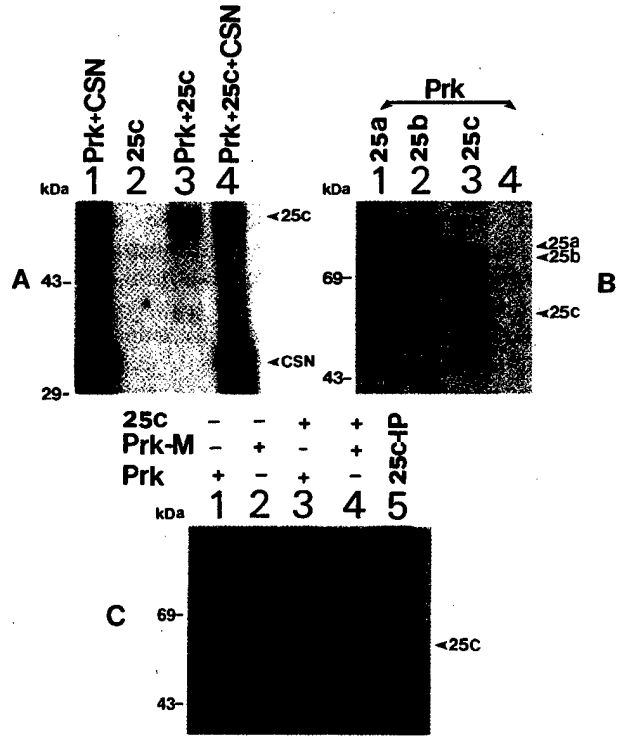

Figure 2 Phosphorylation of His6-Cdc25C by His6-Prk. (**a**) Purified His6-Prk was assayed for its *in vitro* kinase activity towards Cdc25C (2.5 μg/reaction) and casein (15 μg/reaction) in the presence of [γ-^{32}P]ATP. The reaction mixtures were fractionated on a denaturing polyacrylamide gel followed by autoradiography. (**b**) An equal amount (5 μg/reaction) of Cdc25A, GST-Cdc25B, and Cdc25C were phosphorylated *in vitro* by His6-Prk. (**c**) Phosphorylation of Cdc25C by His6-Prk or His6-Prk^{K52R} (Prk-M). Lane 5 (25C-IP) represents Cdc25C immunoprecipitated from ^{32}P-labeled PC-3 cell lysates

residual kinase activity (about 1/5 of His6-Prk, data not shown). It appears that a small fraction of His6-Cdc25C was hyper-phosphorylated and migrated slightly more slowly (Figure 2c, lane 3) as compared with the Cdc25C immunoprecipitated from metabolically labeled Cdc25C (Figure 2c, lane 5).

Many physiological substrates of protein kinases form a relatively stable complex with the enzymes, which can be detected by co-immunoprecipitation. To determine whether Prk and Cdc25C proteins directly interact with each other, we immunoprecipitated protein lysates from *sf*-9 cells infected with His6-Prk or His6-Cdc25C or both recombinant baculoviruses. Immuno-precipitates were analysed by SDS–PAGE followed by Western blotting using the anti-Prk antibody. Figure 3a and b show that His6-Cdc25C immunoprecipitated from double-infected cells (lane 4), but not from cells infected with the wild-type, His6-Prk or His6-Cdc25C baculovirus (lanes 1–3), contained Prk antigen. Further experiments were performed in which various cell lysates were immunoprecipitated with the anti-Prk antibody or control antibodies (anti-Cdc25C as a positive control or anti-β-actin as a negative control). Immunoprecipitates were then analysed for the presence of Cdc25C antigen by Western blotting. It was observed (Figure 3c) that the anti-Prk antibody (lane 1) but not the control antibody (lane 5) brought down Cdc25C antigen. The same blot was stripped and reprobed with the anti-Prk antibody, and it was shown that immunoprecipitates of Cdc25C (Figure 3d, lane 3) but not β-actin (lane 5) contained the Prk antigen.

To ascertain whether Prk interacts with native Cdc25C, we expressed the C-terminal half of Prk as a GST-fusion protein. GST-Prk, as well as control

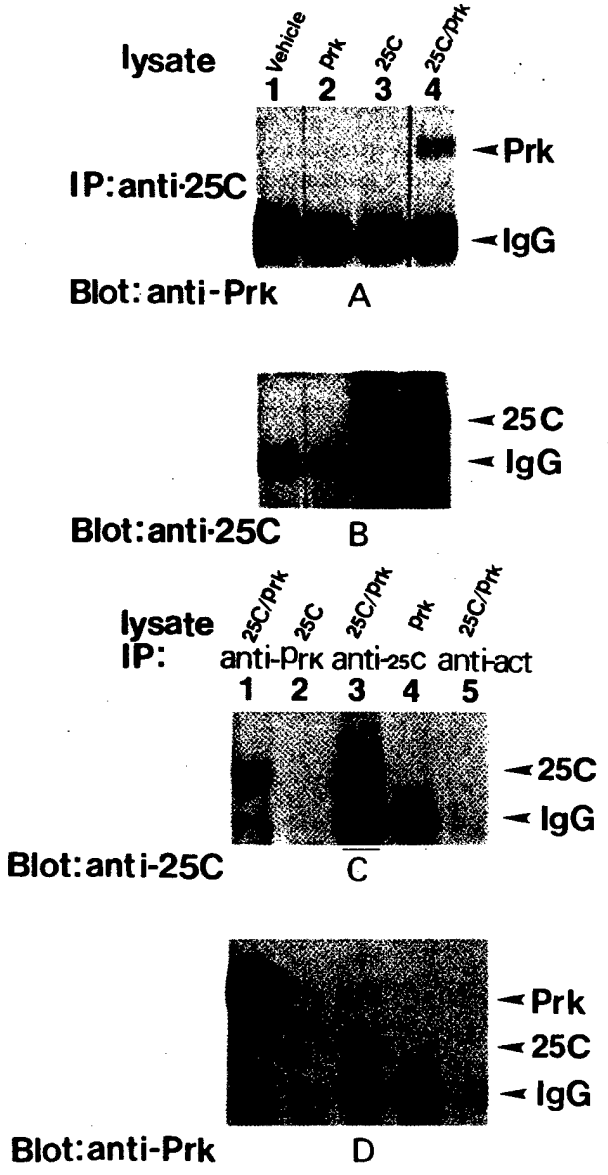

Figure 3 Physical association between His6-Prk and His6-Cdc25C. (**a**) Various *sf*-9 cells infected with the wild-type baculovirus (vehicle), His6-Prk, His6-Cdc25C, or His6-Prk/His6-Cdc25C recombinant baculoviruses were immunoprecipitated with the anti-Cdc25C antibody. Cdc25C immunoprecipitates were analysed by Western blotting using the anti-Prk antibody. (**b**) The blot as shown in **a** was stripped and reprobed with the anti-Cdc25C antibody. (**c**) Various *sf*-9 cell lysates were immunoprecipitated with the anti-Prk (lanes 1 and 2), the anti-Cdc25C (lanes 3 and 4), or the anti-β-actin (lane 5) antibody. Immunoprecipitates were analysed for Cdc25C expression through Western blotting. (**d**) The same blot as shown in **c** was stripped and reprobed with the anti-Prk antibody

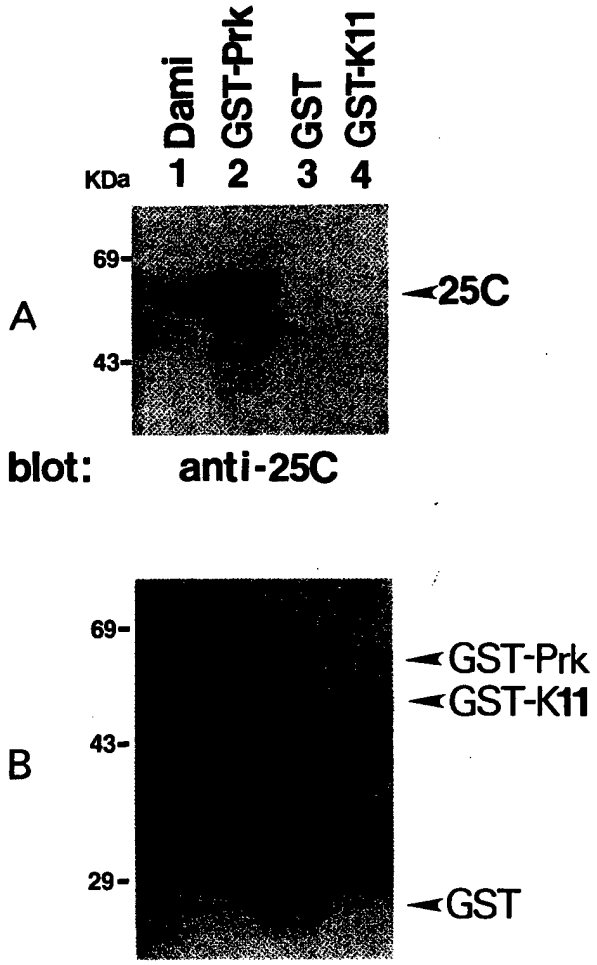

Figure 4 Physical association of GST-Prk with native Cdc25C molecule from Dami Cells. (**a**) GST-Prk proteins as well as control proteins GST or GST-K11 were immobilized on to glutathione resins which were subsequently incubated with protein lysates from Dami cells. After thorough washes, proteins eluted from GST-Prk, GST alone, or GST-K11 were analysed for the presence of the Cdc25C antigen through Western blotting. (**b**) The same blot as shown in **a** was stained with Coomassie brilliant blue

proteins GST-K11 (an RNA-binding factor) or GST, were immobilized to glutathione Sepharose resins as described in the Experimental procedures. Cell lysates (1×10^8 cell equivalent) of Dami cells were incubated with the immobilized GST-Prk, GST-K11, or GST protein (50 μg each). After incubation, the Sepharose resins were thoroughly washed. Proteins bound to GST-Prk, GST-K11 or GST resins were eluted and analysed by SDS–PAGE followed by Western blotting using the anti-Cdc25C antibody. Figure 4a shows that Cdc25C antigen was detected in GST-Prk eluent (lane 2), but not in the eluent of GST alone (lane 3) or GST-K11 (lane 4), indicating that Cdc25C specifically interacts with the C-terminal half of Prk. To confirm that approximately equal amounts of each 'bait' protein were used in the binding, the same blot was stained with Commassie brilliant blue (Figure 4b). It was noted that roughly equal amounts of GST-Prk, GST, or GST-K11 protein were used in the binding although GST-Prk and GST-K11 (Figure 4b, lanes 2 and 4) were partially degraded.

To further determine whether endogenous Prk and Cdc25C interact with each other at a physiological concentration, Cdc25C immunoprecipitates from PC-3 cells were analysed for the presence of Prk antigen by Western blotting. Figure 5a shows that anti-Cdc25C antibody (lane 2) but not a control antibody (lane 1) brought down the Prk antigen. We were unable to detect Cdc25C antigen in Prk immunoprecipitates via Western blotting. We suspected that the anti-Prk antibody may somehow interfere with Cdc25C's association with Prk, and thus reduce interaction efficiency of Cdc25C protein with Prk during immunoprecipitation. To increase detection sensitivity, we metabolically labeled PC-3 cells with ^{32}P-phosphate. Immunoprecipitation of labeled lysates shows (Figure 5b) that the anti-Prk antibody, but not the anti-β-actin antibody (data not shown), is capable of bringing down a protein co-migrating with *in vitro* phosphorylated His6-Cdc25C (lane 1) or Cdc25C immunoprecipitated from PC-3 cell lysates (lane 3). This suggests that Cdc25C physically interact with Prk.

The question of whether Prk phosphorylates Cdc25C was further studied by immunoprecipitating His6-Cdc25C from various *sf*-9 cell lysates using an anti-Cdc25C antibody. The immunoprecipitates were analysed by Western blotting for Cdc25C expression. It was observed that there was a new Cdc25C antigen, with a slow mobility, in doubly-infected cell lysates (Figure 6a, lanes 4 and 6). This Cdc25C antigen was not present in *sf*-9 cells infected with Cdc25C baculovirus alone (Figure 6a, lane 3), suggesting the slow-mobility Cdc25C antigen is a phosphorylation product of Prk. A pretreatment of cell lysates with protein phosphatase converted the slow-moving bands into the fast-moving one (data not shown). To confirm that Cdc25C is associated with Prk *in vivo* and that Cdc25C-associated kinase is capable of phosphorylating the protein phosphatase, His6-Cdc25C immunoprecipitated from equal amounts of cell lysates co-infected with His6-Prk or with His6-Prk^{K52R} baculoviruses was directly incubated with [γ^{32}P]ATP in the kinase buffer. After incubation, the reaction mixture was analysed by SDS–PAGE followed by autoradiography. Figure 6b shows that His6-Cdc25C was phosphorylated strongly in cells co-infected with His6-Prk baculoviruses (lane 1) but weakly in cells co-infected with His6-Prk^{K52R} baculoviruses (lane 3).

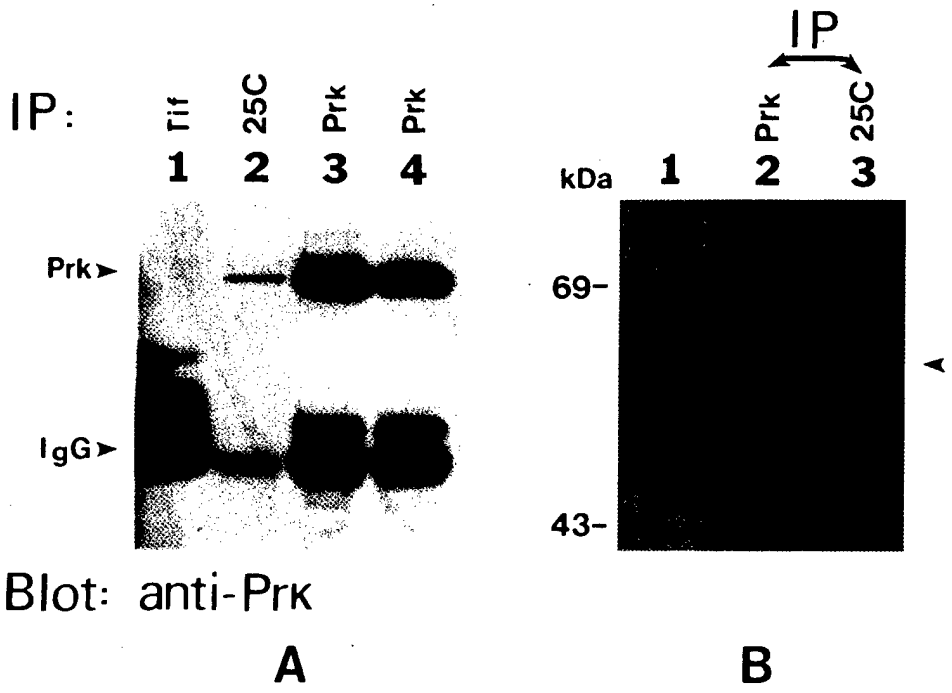

Figure 5 Prk interacts with endogenous Cdc25C. (**a**) PC-3 cell lysates were used for immunoprecipitation with an anti-*tif* (a polyclonal antibody against receptor tyrosine kinase *tif* (lane 1), anti-Cdc25C (lane 2), and anti-Prk (lanes 3 and 4, replicates) antibodies. Immunoprecipitates were blotted for the presence of Prk antigen. (**b**) ^{32}P-labeled PC-3 cell lysates were immunoprecipitated with an anti-Prk (lane 2) or anti-Cdc25C (lane 3) antibody. Immunoprecipitates were fractionated on a SDS polyacrylamide gel followed by autoradiography. Lane 1 represents His6-Cdc25C protein phosphorylated *in vitro* in the presence of [γ^{32}P]ATP by His6-Prk as a standard

Western blotting showed that both His6-Prk and His6-Prk^{K52R} were expressed at similar levels in these cells (data not shown). This suggests that Prk was the primary kinase phosphorylating His6-Cdc25C. Additional experiments were also performed in which various *sf*-9 cell lysates were immunoprecipitated with the anti-Prk antibody, and Prk immunoprecipitates were analysed for kinase activity without addition of exogenous substrates. It was observed that His6-Prk, immunoprecipitated from doubly-infected *sf*-9 cells or the cells infected with His6-Prk baculovirus alone, was autophosphorylated (Figure 6c, lanes 3 and 4). As expected, a protein migrating at about 60 kDa was co-immunoprecipitated with, and phosphorylated by His6-Prk in doubly-transfected (25C/Prk, lane 4). No such protein was present in cells infected with the wild-type (lane 1), His6-Prk (lane 3), or His6-Cdc25C (lane 2) baculovirus alone, strongly suggesting that Cdc25C is an *in vivo* substrate of Prk.

To determine whether Prk phosphorylates Cdc25C at physiologically relevant sites, Cdc25C purified from metabolically-labeled asynchronized PC3 cells, as well as His6-Cdc25C phosphorylated *in vitro* by His6-Prk, were subjected to peptide mapping as described in the Experimental procedures. Figure 7a shows that the phosphopeptide map of His6-Cdc25C phosphorylated by His6-Prk *in vitro* yielded two discrete spots. This map was similar to the one obtained with Cdc25C phosphorylated *in vivo* (Figure 7a). Mixing experiments showed that the major spot from *in vitro* experiments superimposed with the spot of *in vivo* experiments, strongly suggesting that His6-Prk phosphorylated His6-Cdc25C at a site that is also phosphorylated *in vivo*. It is known that the major phosphorylation site on Cdc25C in asynchronized cells is serine^{216} (Ogg *et al*., 1994). Therefore, we used an equal amount of purified mutant GST-Cdc25C^{S216A}(200–256) peptide motif with serine^{216} converted to alanine as well as the wild-type GST-Cdc25C(200–256) peptide motif as *in vitro* substrates of His6-Prk. These peptides were originally used for assays for Chk1 and Chk2 protein kinase activities as reported (Sanchez *et al*., 1997; Matsuoka *et al*., 1998). Figure 7b shows that His6-Prk phosphorylated very poorly, if any, GST-Cdc25C^{S216A}(200–256) *in vitro* (lanes 2 and 3) whereas it strongly phosphorylated GST-Cdc25C(200–256) (lane 1). Moreover, His6-Prk^{K52R} only weakly phosphorylated the wild-type GST-Cdc25C(200–256) motif (Figure 7, lane 4), indicating that Prk phosphorylated Cdc25C on serine^{216}.

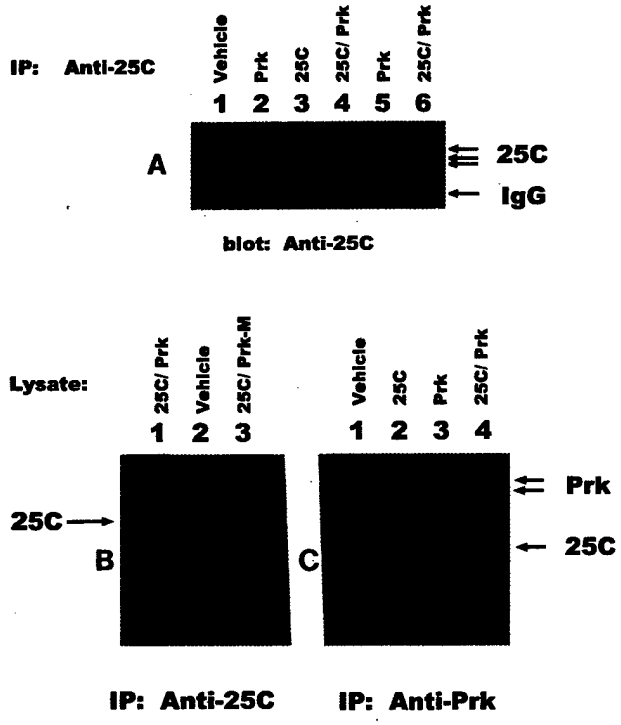

Figure 6 Phosphorylation of His6-Cdc25C by His6-Prk *in vivo*. (**a**) Various *sf*-9 cell lysates were immunoprecipitated with the polyclonal anti-Cdc25C antibody, and the immunoprecipitates were analysed by Western blotting using the monoclonal anti-Cdc25C antibody. Lanes 5 and 6 are duplicates of lanes 2 and 4 from independent experiments. (**b**) *sf*-9 cells doubly-infected with His6-Cdc25C and His6-Prk baculoviruses (lane 1), or with His6-Cdc25C and His6-Prk^{K52R} baculoviruses (lane 3), were lysed and equal amounts of cell lysates were immunoprecipitated with the anti-Cdc25C antibody. Immunoprecipitates were directly analysed for protein kinase activities in the presence of [γ^{32}P]ATP without addition of exogenous substrates. The kinase reaction mixtures were fractionated on denaturing polyacrylamide gel followed by autoradiography. (**c**) Various *sf*-9 cell lysates were immunoprecipitated with the anti-Prk antibody (lanes 1–4). The immunoprecipitates were subjected to *in vitro* kinase assays without addition of exogenous substrates followed by SDS–PAGE and autoradiography as described in B

Discussion

Several previously characterized protein kinases have been implicated in phosphorylating and regulating the activity of Cdc25C. It has been shown that Plx1, a *Xenopus* Plk, physically associates with and phosphor-

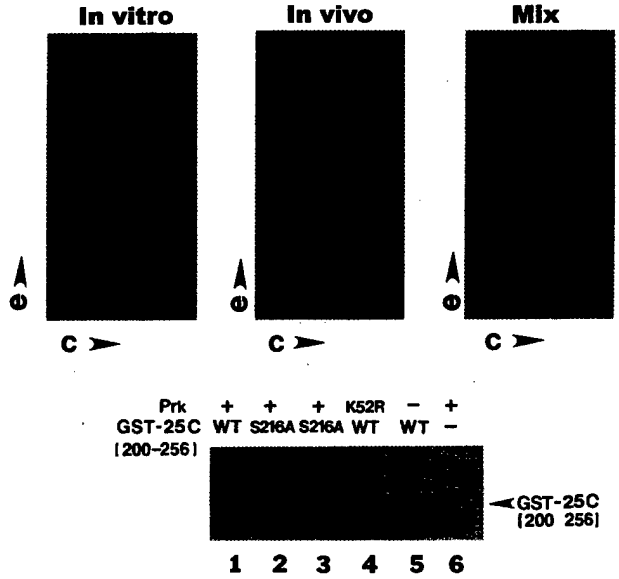

Figure 7 His6-Prk phosphorylates His6-Cdc25C on serine^{216}. (**a**) His6-Cdc25C protein phosphorylated *in vitro* by His6-Prk and Cdc25C protein purified from ^{32}P-labeled PC-3 cell lysates were subjected to peptide mapping as described in the Experimental procedures. The loading origins were marked as O. (**b**) Equal amounts of purified GST-Cdc25C(200–256) and GST-Cdc25C^{S216A} (200–216) fusion proteins were used as *in vitro* substrates for His6-Prk or His6-Prk^{K52R} in the presence of [γ^{32}P]ATP. The *in vitro* phosphorylated samples were analysed by SDS–PAGE followed by autoradiography

ylates Cdc25 protein (Kumagai and Dunphy, 1996). The Plx1-phosphorylated Cdc25 has an enhanced activity towards its substrate $p34^{cdc2}$ (Kumagai, and Dunphy, 1996), suggesting that Plx1 is a regulator for Cdc25. On the other hand, it has been demonstrated that Plk1 may be a substrate of $p34^{cdc2}$ and be involved in regulating the activity of anaphase-promoting complex (Kotani *et al.*, 1998), and that Plx1 is essential for degradation of a mitotic regulator and for exit from M phase (Descombes and Nigg, 1998). These observations suggest that Plk1 may have an additional, if not the primary, role during the progression of mitosis. Recently, it has been shown that c-Tak1, as well as the DNA damage checkpoint kinases Chk1 and Chk2, phosphorylate Cdc25C on $serine^{216}$ (Peng *et al.*, 1997, 1998; Matsuoka *et al.*, 1998). Phosphorylation of Cdc25C on $serine^{216}$ by *chk1* and *chk2* gene products apparently does not directly modulate its phosphatase activity, instead it promotes 14–3–3 protein binding (Sanchez *et al.*, 1997; Peng *et al.*, 1997). 14–3–3 protein then sequestrates Cdc25C in cytoplasm resulting in down-regulation of Cdc25C enzymatic activity *in vivo* (Lopez-Girona *et al.*, 1999).

We and others have previously shown that *prk* (Ouyang *et al.*, 1997) and *plk* (Lee and Erikson, 1997) genes are functional homologues of the budding yeast CDC5. We have also demonstrated that Prk's kinase activity is regulated during the cell cycle peaking at late S and G2 stages although M phase cells also contain a considerable amount of Prk activity (Ouyang *et al.*, 1997). In this paper, we report that purified His6-Prk, but not $His6\text{-}Prk^{K52R}$, is capable of strongly phosphorylating His6-Cdc25C *in vitro*. Using the co-immunoprecipitation approach, we have shown that His6-Prk interacts with His6-Cdc25C expressed in *sf*-9 cells as well as with native Cdc25C from PC-3 cells. In addition, His6-Prk is capable of phosphorylating His6-Cdc25C molecules *in vitro* at a site that is also phosphorylated *in vivo* in asynchronized cells. Considering that the activity of Cdc25C is regulated via reversible phosphorylation (Izumi and Maller, 1995; Kumagai and Dunphy, 1996), our current studies strongly suggest that Prk is a cellular regulator for Cdc25C.

It is intriguing to note that His6-Prk phosphorylates His6-Cdc25C *in vitro* on $serine^{216}$ (Figure 7). This is a site whose phosphorylation would lead to inactivation of Cdc25C activity due to sequestration in cytoplasm by 14–3–3 (Lopez-Girona *et al.*, 1999), which is apparently inconsistent with Prk's role in complementation of CDC5 mutants (Ouyang *et al.*, 1997). However, one likely explanation is that Prk may be involved in down-regulation of $p34^{cdc2}$ activity during late mitosis because considerable Prk activities remain during mitosis (Ouyang *et al.*, 1997). It has been established that activation of $p34^{cdc2}$ is controlled not only by its association with cyclin B but also by reversible phosphorylation. It is possible that inactivation of $p34^{cdc2}$ at late mitosis is also controlled by multiple mechanisms. Namely, in addition to degradation of cyclin B, inactivation of $p34^{cdc2}$ is reinforced by sequestration in cycoplasm of Cdc25C that has been phosphorylated on $serine^{216}$ by kinases such as Prk and perhaps Plk. This notion is, in fact, consistent with the observation that CDC5 *ts* mutants are arrested at late mitosis, a phenotype similar to accumulation of non-degradable cyclin B in mammalian cells. It is also consistent with the recent observation that Plx1 is required for exit from mitosis in *Xenopus* (Descombes and Nigg, 1998).

An alternative explanation of $serine^{216}$ phosphorylation of Cdc25C by Prk is that the kinase may be regulated by DNA damage checkpoint because CDC5 from the budding yeast participates in adaptation of DNA-damage-induced cell arrest (Toczyski *et al.*, 1997). One may propose that Prk expression and/or its activity may be down-regulated in response to DNA damage considering that Prk complements CDC5 *ts* mutant (Ouyang *et al.*, 1997) and thus promoting cell cycle progression. Whether or not Prk is down-regulated during DNA damage is under current investigation. On the other hand, signals other than DNA damage may also elicit $serine^{216}$ phosphorylation of Cdc25C. For example, c-Tak-1 kinase is a very active $serine^{216}$ kinase of Cdc25C (Peng *et al.*, 1998). It is yet to be demonstrated whether c-Tak-1 is activated by DNA damage checkpoint. In addition, Cdc25C is phosphorylated during G1, S, and G2 (Ogg *et al.*, 1994) in the absence of DNA damage or un-replicated DNA. Therefore, it is clear that protein kinases other than Chk1 and Chk2 are phosphorylating Cdc25C on $serine^{216}$ during normal progession of the cell cycle.

Materials and methods

Cell culture

Insect *sf*-9 cells were cultured at 27°C in Grace's insect medium supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA) and antibiotics (100 U/ml penicillin, 50 μg/ml streptomycin sulfate, and 50 μg/ml gentamicin, GIBCO/BRL). Dami (megakaryoblastic, ATCC, Rockville, MD, USA) and PC-3 (prostatic carcinoma, ATCC) cells were grown in RPMI1640 medium, supplemented with 10% FBS, at 37°C with 5% CO_2.

GST-Fusion protein expression and Western blotting

GST-Cdc25A, GST-Cdc25B, $GST\text{-}p34^{cdc2}$ and Prk (amino acids 334–607, Ouyang *et al.*, 1997) were expressed as glutathione *s*-transferase (GST) fusions. Fusion proteins were induced by isopropyl-β-D-thiogalactopyranoside (IPTG) and purified through affinity column chromatography using glutathione sepharose resins according to the protocol provided by the supplier. GST-Cdc25C (200–256) and its mutant $GST\text{-}Cdc25\ C^{S216A}$ (200–256) fusion proteins were kindly provided by Yolanda Sanchez. Mouse monoclonal anti-Prk antibody was produced against an epitope in the C-terminal half of Prk (Ouyang *et al.*, 1997). Mouse monoclonal anti-Cdc25C antibody was produced using the full length of Cdc25C as an immunogen. Both antibodies are available from PharMingen Inc. (San Diego, CA, USA). A rabbit polyclonal anti-Cdc25C antibody was purchased from Santa Cruz Biotech Inc. (Santa Cruz, CA, USA). Western blot analyses were described previously (Ouyang *et al.*, 1997).

Expression and purification of recombinant human Prk

Full-length recombinant Prk was expressed using the baculoviral expression system (PharMingen) following the manufacturer's protocol. Briefly, a cDNA fragment containing the entire open reading frame of human *prk* was cloned into PVL-1393 transfer vector. To facilitate purification of recombinant Prk protein, a short nucleotide sequence coding for six histidine residues was inserted in-frame immediately

after the ATG codon of *prk* cDNA. The baculoviral expression vector BaculoGold™ DNA and the transfer plasmid PVL-1393-Prk were then co-transfected into insect *sf*-9 cells. A cDNA encoding a kinase-defective Prk (Prk^{K52R}) was generated using a site-directed mutagenesis kit (GIBCO/BRL). His6-Prk^{K52R} was expressed in and purified from *sf*-9 cells in the same manner as His6-Prk. Recombinant baculoviruses expressing His6-Cdc25C and GST-Wee1 were kindly provided by Dr Piwnica-Worms (Howard Hughes Medical Institute, Washington University School of Medicine, USA). Insect *sf*-9 cells expressing recombinant His6-Prk and His6-Cdc25C were harvested and lysed for analysis or for purification of recombinant proteins using Ni-NTA resins (Qiagen, Chatworth, CA, USA).

Immunoprecipitation and protein kinase assays

Immunoprecipitation and immunocomplex kinase assays were essentially as described (Ouyang *et al.*, 1997). Purified recombinant His6-Prk or His6-Prk^{K52R}, expressed through the baculoviral expression system, was assayed for its *in vitro* kinase activity using substrates such as casein (15 μg/reaction), GST-Cdc25A (5 μg/reaction), GST-Cdc25B (5 μg/reaction) or His6-Cdc25c (5 μg/reaction, unless specified otherwise), GST-Cdc25C (200–256) (1 μg/reaction) or GST-$Cdc25C^{S216A}$ (200–256) (1 μg/reaction). The kinase reaction conditions were the same as described previously (Ouyang *et al.*, 1997).

Metabolic labeling

The PC-3 cells were cultured in RPMI-1640 medium containing 10% FBS to about 80% confluence. After two washings with pre-warmed phosphate-free RPMI-1640 medium, asynchronized PC-3 cells ($\sim 5 \times 10^5$) were pulsed in the fresh phosphate-free RPMI-1640 medium containing 1.5% dialyzed FBS and 2 mCi [^{32}P]phosphate at 37°C for 4 h. After the pulse, cells were washed and lysed in the lysis buffer (Ouyang *et al.*, 1997) containing 3 μM okadaic acid.

Phosphopeptide mapping

Radio-labeled Cdc25C protein was obtained either by phosphorylation *in vitro* by His6-Prk in the presence of ^{32}P or immunoprecipitated from metabolically-labeled PC-3 cell lysates. After supplementation with the carrier protein (200 μg bovine serum albumin) ^{32}P-Cdc25C protein was precipitated by the addition of trichloroacetic acid [15% (v/v) final concentration]. The protein precipitates were washed twice with cold acetone and redissolved in 50 μl NH_4HCO_3 (200 mM) containing 40 μg tosylphenylanyl chloromethyl ketone (TPCK)-treated trypsin. The dissolved protein samples were digested to completion by TPCK-treated trypsin and then dried under vacuum. The digested peptide samples were redissolved in 5 μl running buffer [5.6% (v/v) glacial acetic acid, 2.5% (v/v) formic acid, pH 1.9] and spotted onto cellulose thin layer chromatography (TLC) plates. Peptide fragments were separated first by electrophoresis using a Multiphor II unit (Pharmacia, Piscataway, NJ, USA) and then by chromatography as described (Boyle *et al.*, 1991). The TLC plates were autoradiographed.

Acknowledgements

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Reactive Oxygen Species-induced Phosphorylation of p53 on Serine 20 Is Mediated in Part by Polo-like Kinase-3*

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Upon exposure of cells to hydrogen peroxide (H_2O_2) phosphorylation of p53 was rapidly induced in human fibroblast GM00637, and this phosphorylation occurred on serine 9, serine 15, serine 20, but not on serine 392. In addition, H_2O_2-induced phosphorylation of p53 was followed by induction of p21, suggesting functional activation of p53. Induction of phosphorylation of p53 on multiple serine residues by H_2O_2 was caffeine-sensitive and blocked in $ATM^{-/-}$ cells. Polo-like kinase-3 (Plk3) activity was also activated upon H_2O_2 treatment, and this activation was ATM-dependent. Recombinant His_6-Plk3 phosphorylated glutathione *S*-transferase (GST)-p53 fusion protein but not GST alone. When phoshorylated *in vitro* by His_6-Plk3, but not by the kinase-defective mutant His6-$Plk3^{K52R}$, GST-p53 was recognized by an antibody specifically to serine 20-phosphorylated p53, indicating that serine 20 is an *in vitro* target of Plk3. Also serine 20-phosphorylated p53 was coimmunoprecipitated with Plk3 in cells treated with H_2O_2. Furthermore, although H_2O_2 strongly induced serine 15 phosphorylation of p53, it failed to induce serine 20 phosphorylation in Plk3-dificient Daudi cells. Ectopic expression of a Plk3 dominant negative mutant, $Plk3^{K52R}$, in GM00637 cells suppressed H_2O_2-induced serine 20 phosphorylation. Taken together, our studies strongly suggest that the oxidative stress-induced activation of p53 is at least in part mediated by Plk3.

Reactive oxygen species (ROS),[1] ubiquitously present, are very reactive and cause damage to biological molecules, including DNA. ROS are potentially mutagenic and may be involved in activation of protooncogene and inactivation of tumor suppressor genes (1, 2). Thus, ROS are suspected to represent important human carcinogens (3, 4). Oxidative signals, either external or internal, are thought to be detected by sensor molecules and mediated by cellular signal transduction systems, which eventually results in cell cycle arrest, senescence, or apoptosis in normal diploid fibroblast cells. ATM has been proposed to be a sensor of oxidative damage of cellular macromolecules such as DNA (5). The tumor suppressor protein p53 appears to be a major effector of the genotoxic stress-signaling pathway that is mediated by ATM (6). In fibroblast cells, p53 protein level is increased upon H_2O_2 treatment, and the level of p53 is correlated with replicative senescence and apoptosis (7). In the $p66shc^{-/-}$ cells, p53 activation and its target gene p21 expression are impaired in response to oxidative stress (8). However, a p53-independent pathway that mediates H_2O_2-induced G_2/M growth arrest has also been reported (9).

Members of the Polo family of protein kinases, conserved through evolution, have been characterized in yeast (10), *Caenorhabditis elegans* (11), *Drosophila melanogaster* (12), *Xenopus laevis* (13), mouse (14, 15), and human (16, 17). The founding member of this family, Polo, was originally identified in the fruit fly and was shown to be a serine-threonine kinase required for mitosis (12). Mammalian cells contain at least three proteins (Plk1, Plk2, and Plk3) that exhibit marked sequence homology to Polo (14, 15, 18, 19). As cells progress through the cell cycle, Plk proteins undergo substantial changes in abundance, kinase activity, or subcellular localization. In human cells, the amounts of Plk1 protein and its kinase activity peak at mitosis (18). During mitosis, Plk1 transiently associates with mitotic structures such as the spindle apparatus, kinetochores, and centrosomes (20). Recent studies have shown that Plk1 contributes to a variety of mitotic (or meiotic) events, including activation of cyclin B-Cdc2, breakdown of the nuclear membrane, centrosome maturation, and formation of the bipolar spindle at the onset of mitosis (21–23). Plk1 also controls the exit of cells from mitosis by regulation of the anaphase-promoting complex (24). Plk3 shows little resemblance to Plk1 with regard to function in mammalian cell cycle regulation. Thus, the abundance of Plk3 remains relatively constant during the cell cycle, and its kinase activity peaks during late S and G_2 phases (25). Furthermore, Plk3 phosphorylates Cdc25C on serine 216, resulting in inhibition of the activity of this protein (25), whereas phosphorylation of Cdc25C by Plx1, a *Xenopus* Plk1 ortholog, results in activation of this protein (13).

Polo family kinases also participate in the response to DNA damage (26–28). For example, Cdc5, a budding yeast ortholog of *Drosophila* polo, promotes adaptation to cell cycle arrest at the DNA damage checkpoint (29). The electrophoretic mobility of Cdc5 in denaturing gels is affected by prior subjection of cells to DNA damage, and this modification is dependent on Mec1, Rad53 (a yeast Chk1 homolog), and Rad9 (26). In addition, a functionally defective Cdc5 mutant protein suppresses a Rad53 checkpoint defect, whereas overexpression of Cdc5 overrides checkpoint-induced cell cycle arrest (27), suggesting that Cdc5 acts downstream of Rad53. Moreover, DNA damage appears to interfere with the activation of Plk1 in mammalian cells, resulting in down-regulation of the kinase activity of this protein. In contrast, expression of dominant negative mutants of Plk1

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[1] The abbreviations used are: ROS, reactive oxygen species; IR, ionizing radiation.

overrides the induction of G_2 arrest by DNA damage (28).

We have been studying the biological role of polo-like kinase-3 (Plk3, previously named Prk) during normal and abnormal cell growth (17, 25, 30, 31). Here we report that ROS induces activation of Plk3 as well as p53, which is correlated with p53 phosphorylation on multiple serine sites. Activation of both Plk3 and p53 is ATM-dependent. In addition, we have obtained experimental evidence strongly suggesting that Plk3 mediates ROS-induced serine 20 phosphorylation of p53.

MATERIALS AND METHODS

Cell Culture—Various cell lines, including ATM-deficient cell line (ATCC number CRL-1702), were purchased from ATCC. CRL-1702 has been characterized as $ATM^{-/-}$ (32). GM00637 cell line (human fibroblast) was originally from the Coriell Institute for Medical Research. HeLa, A549, GM00637, DU145, LNCap, and PC-3 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and antibiotics (100 μg/ml penicillin and 50 μg/ml streptomycin sulfate) with 5% CO_2. DAMI, HEL, and HL-60 cells were cultured in RPMI 1640 medium, and Daudi cells were culture in McCoy's medium supplemented with fetal bovine serum and antibiotics as above.

Immunoblotting—Cells treated with H_2O_2 (200 μM unless otherwise specified) or adriamycin (100 μM) were collected and lysed (25). In some experiments, caffeine (2 μM) was supplemented to the cultured cells for 30 min prior to the treatment with H_2O_2 or adriamycin. Equal amounts (40 μg) of protein lysates from the treated cells were analyzed by SDS-polyacrylamide gel electrophoresis followed by immunoblotting with antibodies (New England Biolabs) to phosphorylated p53 (specifically phosphorylated on serine 9, serine 15, or serine 20), p21, or Bax. The same blots were also stripped and reprobed with antibodies to regular p53 (Santa Cruz Biotechnology). Signals were detected with horseradish peroxidase-conjugated goat secondary antibodies (Sigma) and enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

Protein Kinase Assays—Immunocomplex kinase assays were performed essentially as described previously (25). In brief, A549 cells were exposed to H_2O_2 (200 μM) for various times, lysed, and subjected to immunoprecipitation with antibodies to Plk3. The resulting precipitates were resuspended in a kinase buffer (10 mM Hepes-NaOH (pH 7.4), 10 μM $MnCl_2$, 5 mM $MgCl_2$), and the kinase reaction was initiated by the addition of [γ-^{32}P]ATP (2 μCi) (Amersham Pharmacia Biotech) and α-casein (Sigma). After incubation for 30 min at 37 °C, the reaction mixtures were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. Recombinant His_6-Plk3, produced and purified as described previously (25, 30), was assayed for kinase activity as a positive control. In some kinase assays, GST-p53 was incubated with His_6-Plk3 or His_6-$Plk3^{K52R}$ in the kinase buffer supplemented with "cold" ATP. After reaction, Plk3-phosphorylated GST-p53 samples, as well as nonphosphorylated GST-Plk3, were blotted for serine 20 phosphorylation.

Coimmunoprecipitation Analysis—GM00637 cell lysates were incubated for 30 min at room temperature in a total volume of 500 μl with of 20 μl of protein A/G-agarose bead slurry (Santa Cruz Biotechnology). After removal of the beads, the supernatant was supplemented with either rabbit polyclonal (PharMingen) or mouse monoclonal antibodies to Plk3, or with control immunoglobulins followed by incubation with constant agitation for overnight at 4 °C. Protein A/G-agarose beads (20 μl) were then added to each immunoprecipitation mixture, and the incubation was continued for 1 h at room temperature. Immunoprecipitates were collected by centrifugation, washed three times with the cell lysis buffer, and subjected to immunoblot analysis with monoclonal antibodies to serine 20-phosphorylated p53.

Transient Transfection—GM00637 cells were transfected, using the LipofectAMINE method (Life Technologies, Inc.), with constructs expressing Plk3 or $Plk3^{K52R25}$ or with the vector pCR592. One day after transfection, cells were treated with or without H_2O_2 for 30 min. Cell lysates were prepared and blotted for Plk3, p53, or serine 20-phosphorylated p53.

RESULTS

Although recent studies have shown that phosphorylation of p53 plays an important role in stabilization and activation of this tumor suppressor protein in cells exposed to ionizing radiation (IR) or UV (6, 33), the mechanism by which ROS-induced p53 accumulation/activation remains unclear (34). To determine whether ROS activates p53 through phosphorylation, GM00637D cells were exposed to H_2O_2 for various times, and p53 phosphorylation status was analyzed by immunoblotting using phospho-specific antibodies. Fig. 1*A* shows that upon H_2O_2 treatment, p53 was rapidly phosphorylated on serine 20 and serine 15 in GM00637D cells. Serine 9 phosphorylation was also induced with a slow kinetics (Fig. 1*A*, *lane 5*). However, serine 392 phosphorylation was not detected (data not shown). These results indicate that p53 accumulation upon oxidative stress as reported by von Harsdorf and Dietz (35) is at least partly due to phosphorylation of p53 on serine 15 and serine 20, because these two residues are located within the domain of the protein that interacts with HDM2 (human ortholog of murine double minute-2 protein, MDM2), resulting in stabilization of the normally short-lived p53 protein in response to the stress (36).

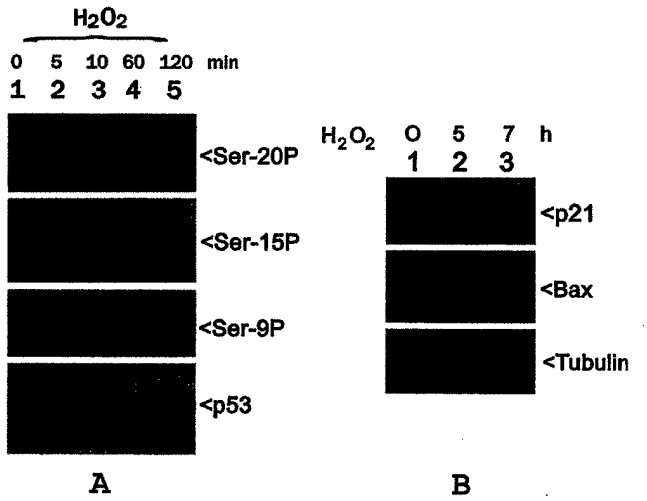

FIG. 1. **H_2O_2 induces phosphorylation and activation of p53.** *A*, GM00637 cells treated with H_2O_2 for the indicated times were collected, and equal amounts of cell lysates were analyzed for p53 phosphorylation via Western blotting using antibodies specific to phosphorylated serine 9, serine 15, or serine 20. The same blot was stripped and reprobed with antibody to all forms of p53. *B*, GM00637D cells were treated with H_2O_2 for the indicated time, and equal amounts of cell lysates were blotted for p21, Bax, or α-tubulin.

Phosphorylation and activation of p53 upon challenge with genotoxic stress such as IR and UV often results in cell cycle arrest (6). In fact, the trans-activation by p53 of genes such as those encoding p21 and Bax proteins is thought to be responsible at least in part for cell cycle arrest and apoptosis, respectively, in cells subjected to genotoxic stress (37). To determine whether H_2O_2-induced p53 phosphorylation is correlated with its functional activation, we measured expression of its target genes p21 and Bax. Fig. 1*B* shows that 5 h after H_2O_2 treatment, the p21 protein level began to increase (*lane 2*) and that 7 h post-treatment it was more than quadrupled (*lane 3*) compared with the untreated control (*lane 1*). On the other hand, little increase in Bax protein levels were observed in cells treated with H_2O_2.

DNA damage caused by IR activates p53 through phosphorylation on multiple residues, and this activation is ATM-dependent (33). To determine whether ROS-induced p53 phosphorylation was also ATM-dependent, we treated GM00637 cells with caffeine, an ATM/ATR inhibitor, prior to exposure of the cells to H_2O_2 or IR-mimetic drug adriamycin. Fig. 2*A* shows that caffeine (*CFN*) partially blocked H_2O_2-stimulated phosphorylation of p53 on serine 15 and serine 20 (*lanes 2* and *5*), whereas it completely inhibited adriamycin (*ADR*)-induced phosphorylation of p53 on all three residues (*lanes 3* and *6*).

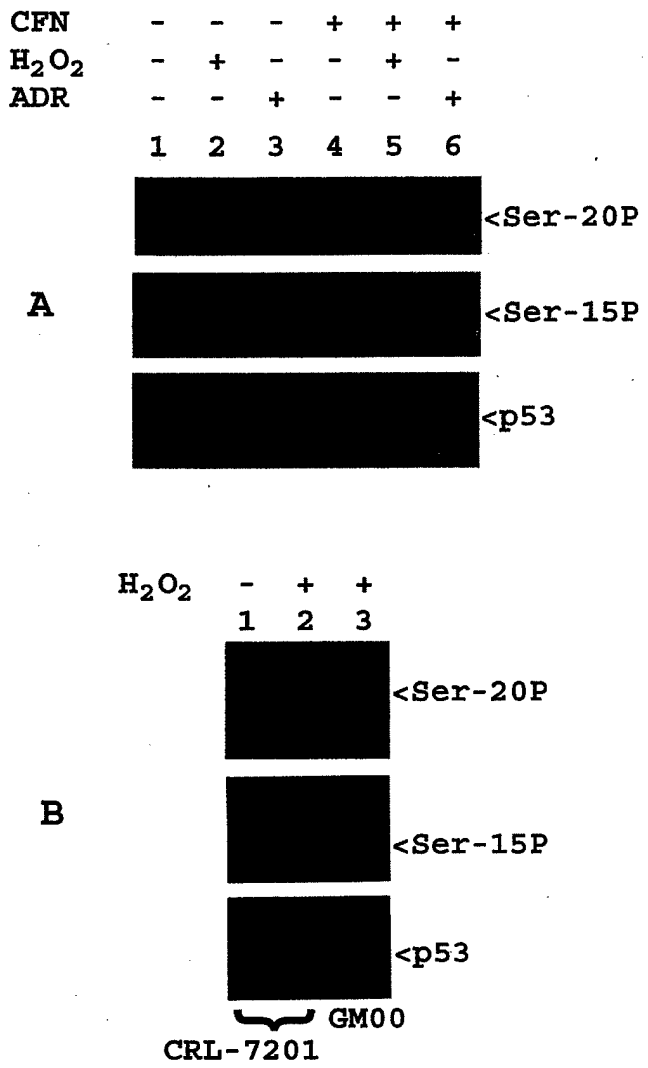

FIG. 2. **H_2O_2-induced p53 phosphorylation is ATM-dependent.** *A*, GM00637 cells pretreated with caffeine (*CFN*) were exposed to H_2O_2 or adriamycin (*ADR*) for 30 min. Equal amounts of protein lysates from the treated cells were analyzed for p53 phosphorylation using phospho-specific antibodies to serine 15 or serine 20. The same cell lysates were also blotted for all forms of p53. *B*, ATM-deficient cells (*CRL-1702*) were treated with H_2O_2 for 30 min, and equal amounts of cell lysates were blotted with antibodies to serine 20-phosphorylated or serine 15-phosphorylated p53, as well as all forms of p53. GM00637 cell lysates (*lane 3*) were used as positive control.

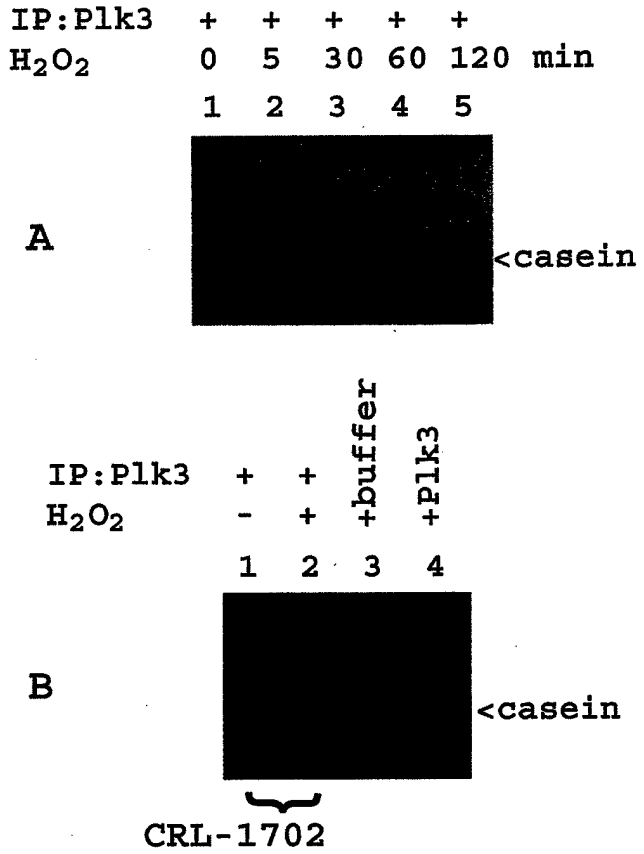

FIG. 3. **H_2O_2 activates Plk3 in an ATM-dependent manner.** *A*, A549 cells treated with H_2O_2 for the indicated times were immunoprecipitated with an antibody to Plk3. Plk3 immunoprecipitates were analyzed for *in vitro* kinase activity using casein as substrate. *B*, Plk3 immunoprecipitated from ATM-deficient cells (*CRL-1702*) treated with (*lane 2*) H_2O_2 for 30 min, as well as the untreated parental cells (*lane 1*), were analyzed for *in vitro* kinase activity. Vehicle (buffer, *lane 3*) or recombinant His_6-Plk3 (*lane 4*) was used as control for the kinase assay.

These observations suggest that p53 activation by ROS is at least in part dependent on ATM and/or ATR. To further confirm that ATM was important in mediating p53 phosphorylation by H_2O_2, we exposed ATM-deficient CRL-1702 cells to H_2O_2. Fig. 2*B* shows that in the ATM-deficient cells after H_2O_2 treatment enhancement in phosphorylation of p53 on neither serine 20 nor serine 15 was observed, indicating that ROS-induced phosphorylation and activation of p53 is ATM-dependent.

Our laboratory has been studying human Plk3, which is involved in regulating cell cycle progression (17, 25, 30). As an initial step to identify protein kinase(s) responsible for phosphorylation of p53 induced by ROS, we examined the possibility of Plk3 activation by H_2O_2, because Plk3 phosphorylates the same residue of Cdc25C (serine 216) as that targeted by Chk1 and Chk2. Chk1 and Chk2 are also reported to phosphorylate p53 on serine 20 (38, 39). A549 cells, expressing good levels of Plk3, were treated with ROS for various times. Plk3 immunoprecipitated from the treated cells was analyzed for its kinase activity using casein as substrate as described previously (25). Fig. 3*A* shows that compared with the control (*lane 1*) Plk3 kinase activity was rapidly activated in A549 cells (*lane 2*) and maintained for at least 1 h. To determine whether Plk3 activation was ATM-dependent, CRL-1702 cells treated with H_2O_2 were collected, and Plk3 immunoprecipitates were assayed for Plk3 kinase activity. Fig. 3*B* shows that whereas recombinant Plk3 phosphorylated casein effectively (*lane 4*), no difference in Plk3 kinase activity was detected between untreated control (*lane 1*) and H_2O_2-treated CRL-1702 cells (*lane 2*), suggesting that Plk3 activation also requires ATM. In addition, Plk3 activation was caffeine-sensitive because pretreatment of A549 cells with caffeine completely blocked activation of Plk3 by H_2O_2 (data not shown).

To determine the possibility that Plk3 was involved in mediating H_2O_2-induced p53 phosphorylation, we screened a dozen cell lines for Plk3 expression. We observed (Fig. 4*A*) that Daudi (B lymphoblastic leukemic cells with wild-type p53 (40)) did not express detectable levels of Plk3, whereas other tested cell lines expressed various levels of this protein. Further analysis with polymerase chain reaction confirmed that no Plk3 expression was detectable in Daudi cells (data not shown). To determine whether the absence of Plk3 expression affected p53 phosphorylation, we analyzed p53 phosphorylation on both serine 20 and serine 15 residues in Daudi cells exposed to H_2O_2. Fig. 4*B* shows that p53 phosphorylation on serine 15 is rapidly induced and maintained for at least 2 h in Daudi cells (*lanes 2–5*). In contrast, no serine 20 phosphorylation was observed. Chk2 is reported to phosphorylate p53 on serine 20 (39). Reprobing the same blot with antibody to Chk2 revealed that

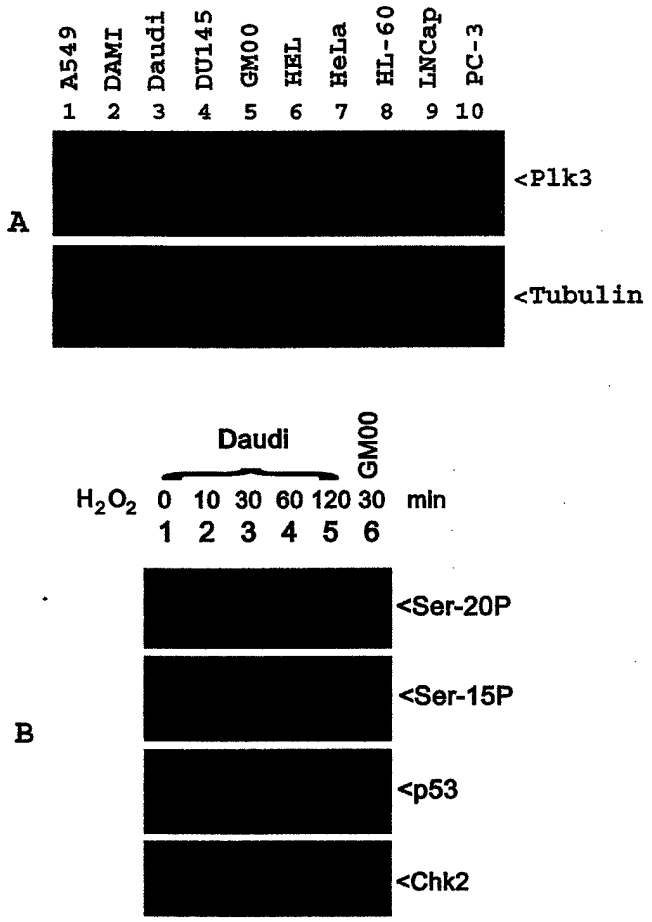

FIG. 4. **Absence of serine 20 phosphorylation in Daudi cells that express no Plk3.** *A*, equal amounts of protein lysates from the indicated cell lines were blotted for both Plk3 and α-tubulin expression. *B*, equal amounts of proteins from Daudi cells treated with H_2O_2 for the indicated times were blotted for p53 phosphorylation using phospho-specific antibody to serine 20 or serine 15 of p53 as well as all forms of p53 and Chk2. GM00637 cell lysates (*lane 6*) were used as a control.

Daudi cells expressed abundant Chk2 (Fig. 4*B*). These observations suggest that Plk3 is involved in regulating serine 20 phosphorylation of p53.

We next asked whether Plk3 directly phosphorylated p53. *In vitro* kinase assays showed (Fig. 5*A*) that recombinant histidine-tagged Plk3 (His_6-Plk3) phosphorylated GST-p53 (*lane 2*), as well as casein (*lane 1*), but not GST alone (*lane 5*), indicating that Plk3 targets the p53 moiety of GST-p53. A kinase-defective mutant of Plk3, His_6-$Plk3^{K52R}$, in which lysine 52 was replaced with arginine, did not significantly phosphorylate GST-p53 (Fig. 5*A*, *lane 3*). To further examine whether the serine 20 residue of p53 was a phosphorylation target of Plk3, we incubated GST-p53 with His_6-Plk3 or His6-$Plk3^{K52R}$ in the kinase buffer supplemented with ATP. *In vitro* phosphorylated GST-p53 samples, as well as nonphosphorylated GST-p53, were blotted for serine 20 phosphorylation. Fig. 5*B* shows that purified GST-p53 was not recognized by the antibody to serine 20-phosphorylated p53 (*lane 4*). However, when phosphorylated *in vitro* by His_6-Plk3, but not by His_6-$Plk3^{K52R}$, GST-p53 exhibited a strong phosphoserine 20 epitope (*lane 5*). Given that Plk3 kinase activity and serine 20 phosphorylation of p53 are induced by H_2O_2, these observations strongly suggest that serine 20 is an *in vivo* target of Plk3 during H_2O_2-induced stress response.

To explore the physical interaction between p53 and Plk3, we immunoprecipitated Plk3 from cells treated with or without H_2O_2, and Plk3 immunoprecipitates were then blotted for the

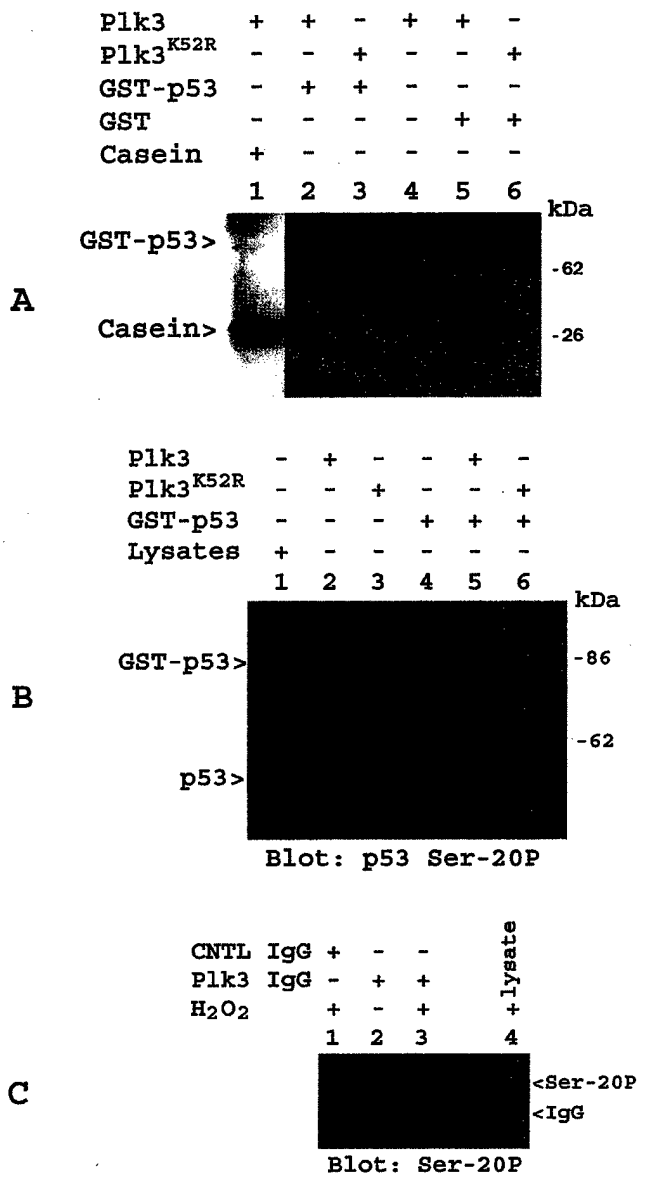

FIG. 5. **Plk3 interacts with and phosphorylates p53 *in vitro*.** *A*, purified GST-p53 was phosphorylated *in vitro* by His_6-Plk3 (*lane 2*) or His_6-$Plk3^{K52R}$ (*lane 3*) in the kinase buffer supplemented with [γ-^{32}P]ATP. GST and α-casein were used as negative and positive controls, respectively. After kinase reaction, samples were fractionated on SDS-polyacrylamide gel electrophoresis followed by autoradiography. *B*, GST-p53 was phosphorylated *in vitro* by His_6-Plk3 and His_6-$Plk3^{K52R}$ in the kinase buffer supplemented with "cold" ATP. The reaction samples and protein lysates from H_2O_2-treated GM00637 cells (*lane 1*) were then blotted with the antibody to phosphoserine 20 of p53. Partial degradation of GST-p53 was observed (*lane 5*). *C*, equal amounts of protein lysates from GM00637 cells treated with or without H_2O_2 were immunoprecipitated with the antibody to Plk3 or control IgGs. Immunoprecipitates were then blotted for serine 20-phosphorylated p53. GM00637 cell lysates were used as a positive control.

presence of serine 20-phosphorylated p53. Fig. 5*C* shows that neither the control IgGs appreciably precipitated serine 20-phosphorylated p53 from the H_2O_2-treated cells (*lane 1*) nor Plk3 antibody brought down the phospho-p53 from the untreated control cells (*lane 2*) However, Plk3 antibody precipitated p53 that was phosphorylated on serine 20 from cells treated with H_2O_2.

To further demonstrate that Plk3 regulated serine 20 phosphorylation of p53 *in vivo*, GM00637 cells were transfected with constructs expressing either Plk3 or $Plk3^{K52R}$. One day after transfection, both Plk3 proteins were expressed (Fig. 6*A*,

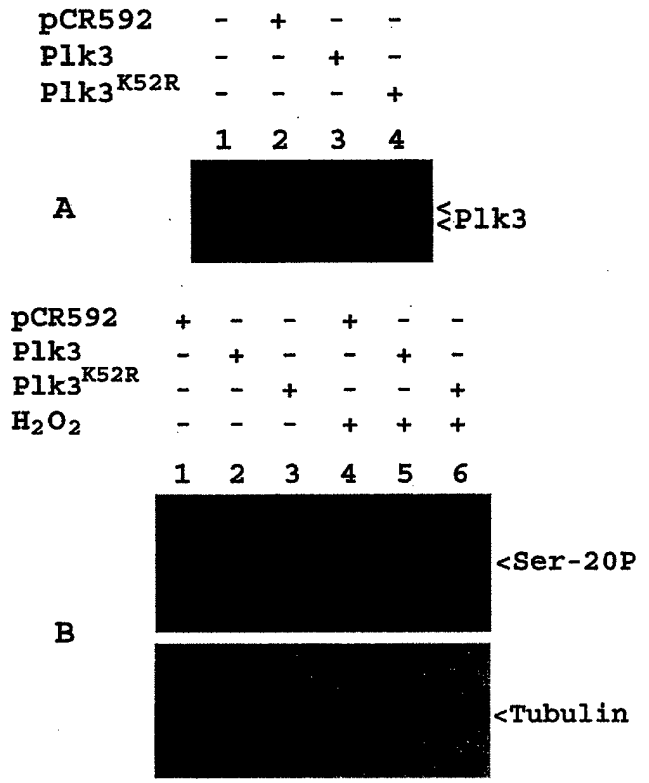

FIG. 6. **Plk3 regulates serine 20 phosphorylation of p53 *in vivo*.** *A*, GM00637 cells were transfected with constructs expressing Plk3 or $Plk3^{K52R}$ or with the vector alone. One day after transfection, cells were lysed, and equal amounts of proteins from the transfected cells were blotted with the antibody to Plk3. *B*, GM00637 cells transfected with various constructs as indicated were treated with or without H_2O_2 (20 μM) for 30 min. Equal amounts of proteins from various treatments were blotted with antibodies to serine 20-phosphorylated p53 or α-tubulin.

lanes 3 and *4*) at a level higher than the endogenous one (the band with a slower mobility). The fast mobility of both transfected Plk3 proteins was due to a short truncation at the amino terminus. Further analysis of the transfected cells showed (Fig. 6*B*) that no significant enhancement in serine 20 phosphorylation was detected when cells were transfected with either Plk3 (*lane 2*) or $Plk3^{K52R}$ (*lane 3*) compared with cells transfected with vector alone (*lane 1*). However, when Plk3-transfected cells were exposed to a low concentration of H_2O_2, a significant increase in serine 20 phosphorylation was detected (*lanes 2* and *5*). In contrast, no such enhancement in serine 20 phsophorylation was detected in cells transfected with $Plk3^{K52R}$ (*lanes 3* and *6*). These observations suggest that Plk3 needs to be activated by ROS before it can fully phosphorylate its physiological substrates.

DISCUSSION

The mechanism by which mammalian cells transmit signals in response to oxidative damage remains unclear. Here we report that ROS phosphorylates and activates p53 tumor suppressor protein. Consequences of p53 activation are either cell cycle arrest or apoptosis. We have observed that p53 activation in response to H_2O_2 treatment results in significant increase in expression of p21, but not of Bax (Fig. 1*B*), which is consistent with our observation that the concentration of H_2O_2 used in our experiments did not cause significant apoptosis of GM00637 cells (data not shown). However, we cannot exclude the possibility of Bax activation by post-translational mechanisms. Interestingly, it has been proposed that p53 may cause cell death by directly stimulating mitochondria to produce an excess amount of toxic ROS in some cells (33). Thus, a feedback loop between p53 and ROS may exist, which is presumably to amplify the stress signal, resulting in accelerated programmed cell death when damage caused by a genotoxic stress is beyond repair.

Recent advances indicate that reversible phosphorylation plays an important role in the DNA damage checkpoint activation. In fact, p53 is rapidly phosphorylated upon exposure of cells to IR or UV (6). Our current studies demonstrated that oxidative stress activates p53 also through phosphorylation on multiple residues. The kinetics of ROS-induced phosphorylation of p53 on various serine residues is apparently different (Fig. 1*A*), suggesting the involvement of several protein kinases. It is also likely that phosphorylation of certain residues may facilitate the subsequent phosphorylation of other residues. Consistent with the latter scenario, phopshorylation of threonine 18 by casein kinase II requires prior phosphorylation of serine 15 by ATM upon DNA damage (41).

Our current studies indicate that Plk3 is directly involved in H_2O_2-induced phosphorylation of p53 on the serine 20 residue. First, induction of both p53 phosphorylation and Plk3 kinase activity by H_2O_2 is ATM-dependent (Figs. 2*B* and 3*B*). Second, H_2O_2 does not induce serine 20 phosphorylation of p53 in Daudi cells that express Chk2 but no detectable levels of Plk3 (Fig. 4). Third, Plk3, but not $Plk3^{K52R}$, directly phosphorylates GST-p53 (but not GST alone) *in vitro*, and Plk3-phosphorylated GST-p53 contains a strong serine 20 epitope (Fig. 5). Fourth, Plk3 interacts with serine 20-phosporylated p53 when cells are exposed to H_2O_2 (Fig. 5*C*). Fifth, ectopic expression of Plk3, but not the kinase-defective mutant $Plk3^{K52R}$, results in significantly enhanced phosphorylation of p53 on serine 20 after H_2O_2 treatment.

Our studies, together with previous observations (30), suggest that Plk3 may act in parallel with Chk1 and Chk2, downstream of ATM or ATR. Plk3 may preferentially transduce signals generated by a specific genotoxic stress such as H_2O_2, just as Chk1 and Chk2 are differentially activated by UV radiation and IR, respectively (6). The observation that serine 20 phosphorylation of p53 was not induced by H_2O_2 in Daudi cells that express abundant Chk2 but no detectable Plk3 supports this notion. On the other hand, given that Cdc5 acts downstream of Rad53 in yeast (26), it is also possible that Plk3 may lie downstream of Chk2 (and/or Chk1). Plk3 may integrate the signals from ATM-Chk2 and ATR-Chk1 and induce cell cycle arrest or apoptosis by phosphorylating either Cdc25C on serine 216 or p53 on serine 20. Consistent with the latter scenario, Plk3 is activated by IR-mimetic drug adriamycin and UV radiation (data not shown) in addition to H_2O_2.

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GENES, CHROMOSOMES & CANCER 27:332–336 (2000)

BRIEF COMMUNICATION

PRK, a Cell Cycle Gene Localized to 8p21, Is Downregulated in Head and Neck Cancer

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The human *PRK* gene encodes a protein serine/threonine kinase of the polo family and plays an essential role in regulating meiosis and mitosis. We have previously shown that *PRK* expression is downregulated in a significant fraction of lung carcinomas. Our current studies reveal that *PRK* mRNA expression is downregulated in a majority (26 out of 35 patients) of primary head and neck squamous-cell carcinomas (HNSCC) compared with adjacent uninvolved tissues from the same patients, regardless of stage. In addition, *PRK* transcripts were undetectable in one of the two HNSCC cell lines analyzed. Ectopic expression of *PRK*, but not a *PRK* deletion construct, in transformed A549 fibroblast cells suppresses their proliferation. Furthermore, fluorescence in situ hybridization analyses show that the *PRK* gene localizes to chromosome band 8p21, a region that exhibits a high frequency of loss of heterozygosity in a variety of human cancers, including head and neck cancers, and that is proposed to contain two putative tumor suppressor genes. Considering that *PRK* plays an important role in the regulation of the G2/M transition and cell cycle progression, our current studies suggest that deregulated expression of *PRK* may contribute to tumor development. *Genes Chromosomes Cancer 27:332–336, 2000.*

In the past few years, an emerging family of protein kinases (designated the polo kinase family) has been described in yeast, *Drosophila*, *Xenopus*, mouse, and humans (Sunkel and Glover, 1988; Simmons et al., 1992; Fenton and Glover, 1993; Kitada et al., 1993; Golsteyn et al., 1994; Hamanaka et al., 1994; Donahue et al., 1995; Li et al., 1996; Nigg, 1998). The polo family kinases are important in regulating the onset of mitosis and M-phase progression (Sunkel and Glover, 1988; Fenton and Glover, 1993; Kitada et al., 1993; Nigg, 1998). Mutants of the polo gene in *Drosophila* induce hypercondensed chromosomes and abnormal spindle formation (Fenton and Glover, 1993). A polo homolog encoded by *CDC5* in budding yeast is required for nuclear division in late mitosis (Kitada et al., 1993) and for adaptation to DNA damage response (Toczyski et al., 1997). Recently, it has been shown that a polo-like kinase (*PLX*) from *Xenopus* interacts with, phosphorylates, and activates the *CDC25* gene product (Kumagai and Dunphy, 1996), a dual specific protein phosphatase that, in turn, activates $p34^{cdc2}$. Significantly, PRK protein shares considerable sequence identity with the *CDC5* gene product and is capable of complementing yeast *CDC5* temperature-sensitive mutants (Ouyang et al., 1997), suggesting that it is a functional homolog of the budding yeast *CDC5*.

Neoplastic disease is characterized by a loss of controlled cell proliferation. Deregulated expression of and/or mutations in cell cycle regulatory molecules such as cyclin D1, $p16^{INK4A}$, and RB1 place cells at risk for neoplastic transformation (Sherr, 1996). Cyclin D1, $p16^{INK4A}$, and RB1 participate in regulating the G1 checkpoint and are critical for maintaining the balance between remaining in G1/G0 and commitment to entry into S-phase. Mutations and aberrant expression of genes regulating stages of the cell cycle other than G1 are also found in human cancers. For example, overexpression of cyclin E or cyclin B1 is detected in breast, stomach, endometrium, and colon carcinomas as well as in acute lymphocytic leukemia (Hunter and Pines, 1994; Kamb, 1995). In addition, TP53, a major tumor suppressor known to regulate the G1/S checkpoint, has recently been shown also to be involved in controlling the G2/M checkpoint (Hermeking et al., 1997). Furthermore, the *CHK1* gene product, a serine/threonine kinase, functions as a key component that links the DNA damage check-

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point to mitotic arrest (Peng et al., 1997; Sanchez et al., 1997). The human *CHK1* gene has been mapped to 11q24 (Peng et al., 1997), a locus that has been shown to contain a potential tumor suppressor (Laake and Odegard, 1997).

We have previously shown that *PRK* expression is downregulated in a significant fraction of lung carcinoma samples (Li et al., 1996), suggesting its potential role in regulation of tumor cell growth. To address whether the *PRK* gene is located at a chromosomal locus linked to known human disease, we first obtained from a λ-phage human genomic library a 15-kbp *PRK* gene fragment that was used for mapping the *PRK* gene via the fluorescence in situ hybridization (FISH) method. FISH was performed essentially as previously described (Heng et al., 1992; Heng and Tsui, 1993). Metaphase-arrested lymphocytes were used for analyses. The hybridization efficiency for the *PRK* probe was about 94%, and consistent signals were restricted to chromosome arm 8p and were not detected at other chromosomal regions under the conditions used. An example of the FISH results is shown in Figure 1A and B. The DAPI banding pattern was recorded separately. The assignment of the FISH mapping data with chromosome bands was achieved by superimposing of the *PRK* signals with the same DAPI-banded chromosome, placing the *PRK* gene at 8p21 (Fig. 1C).

Deletions of chromosome arm 8p, as defined by allelic imbalance, are a frequent event in many types of cancers such as those of prostate, head and neck, lung, and colon. This suggests that there exists one (or more) tumor suppressor gene on this chromosome arm. Considering that the steady-state *PRK* mRNA levels are significantly lower in many lung carcinoma samples than in their normal tissue counterparts from the same patients (Li et al., 1996), we asked whether *PRK* expression was also downregulated in some other tumors such as head and neck squamous-cell carcinoma (HNSCC). To this end, 35 HNSCC samples, collected from patients who underwent surgery between October 1992 and March 1995, were used for analyses. Tissues were obtained immediately after surgery and dissected free of necrotic tissues. One part of each specimen was frozen in liquid nitrogen immediately after removal for further RNA study. Normal tissues were taken at least 2 cm away from the tumor, at the resection margin. All specimens and hematoxylin and eosin-stained histologic slides were reviewed by pathologists to identify normal and tumor areas. Histologic examination confirmed that the specimens were comprised of more than 95% tumor cells. Adjacent normal mucosa was also available from each patient. Normal tissues were also evaluated histologically and found to have at

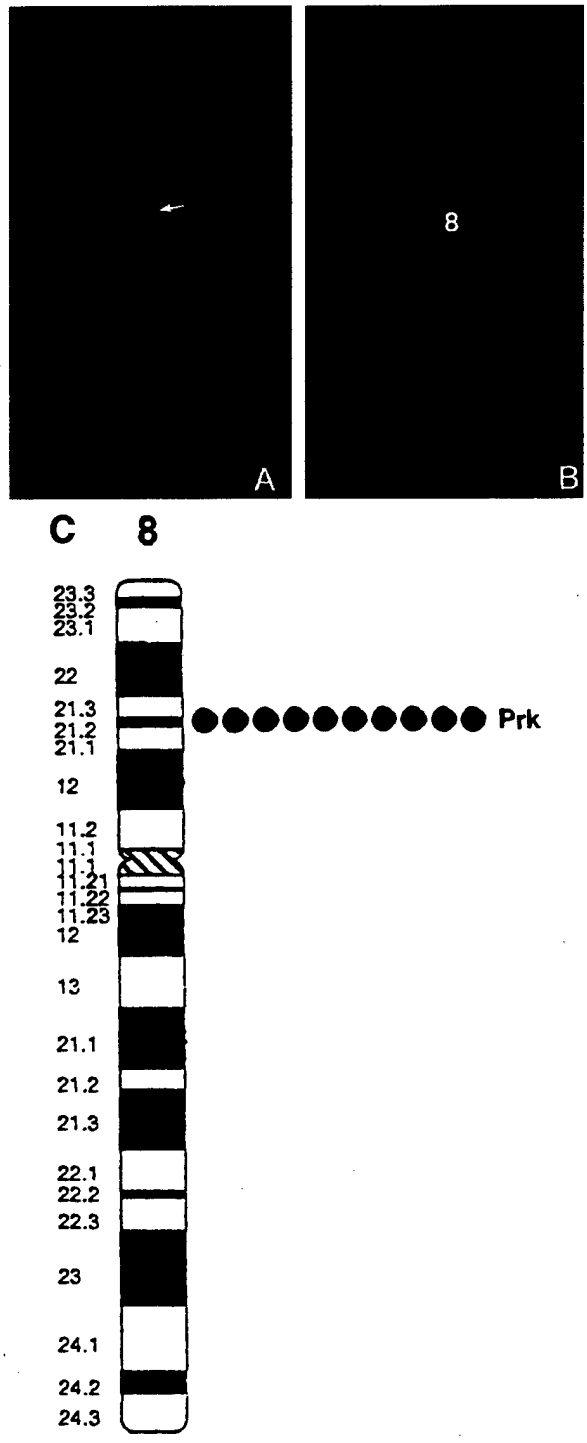

Figure 1. The *PRK* gene localizes to 8p21. **A:** The FISH signals (two yellow dots) on chromosome 8. **B:** The same mitotic figure as shown in A was stained with DAPI, which localizes the *PRK* gene to chromosome 8, subband p21. **C:** Diagram of FISH mapping results for the *PRK* gene. Dots represent the intensity of the signal detected on chromosome 8.

least 95% normal cells. The patients with primary tumors had not received any radiotherapy or chemotherapy. The patients included 26 males and 9 females and had an average age of 56 years (ranging from 31 to 81 years). Of the 35 HNSCC samples used in this study, 30 were primary and 5 were recurrent carcinomas. The primary HNSCCs included 2 stage I, 9 stage II, 11 stage III, and 8 stage IV carcinomas. Tumor sites included the oropharynx (10 patients), oral cavity (10 patients), hypopharynx (2 patients), parapharyngeal space (1 patient), tongue (2 patients), subglottis (1 patient), and supraglottis (9 patients). Histologically, 7 tumors were well differentiated, 24 were moderately differentiated, and 4 were poorly differentiated. Northern blotting analyses revealed that, although the steady-state level of *PRK* mRNA varied greatly among individual patients (Fig. 2A and B), the tumors (odd lanes) consistently expressed less or no detectable *PRK* mRNA levels compared with adjacent uninvolved tissues (even lanes) from the same patients. A summary of *PRK* expression for the 35 HNSCC samples is presented in Table 1. Expression of *PRK* was undetectable in tumor as well as paired uninvolved samples in four patients. Twenty-six tumor samples showed a significant downregulation of the *PRK* mRNA level compared with the uninvolved tissues from the same patients. Downregulation of *PRK* expression does not appear to be associated with tumor stage. Among patients with recurrent tumors, one carcinoma sample showed a significant increase in *PRK* mRNA expression. All specimens were examined for histology, which revealed that although they were comprised predominantly of tumor cells (about 95%), there was some tissue heterogeneity in all samples (data not shown). Many of the tissues samples used in this report were also used for analysis of *TP53* and *RB* expression (Li et al., 1995, 1997).

We further analyzed two squamous-cell carcinoma cell lines derived from head and neck cancers (UN-SCC29 and UM-SCC6), as well as several other tumor-derived cell lines, for *PRK* expression. Cell lines derived from solid tumors included A549 (transformed lung fibroblast, ATCC), Weri-Rb1 (retinoblastoma, ATCC), UM-SCC29 (head and neck carcinoma), UM-SCC6 (head and neck carcinoma), an endothelium tumor-derived cell line (established in-house), a medulla blastoma-derived cell line (established in-house), and HTP-1 (bladder carcinoma, ATCC). UM-SCC6 and UM-SCC27 cell lines were kindly provided by Dr. Thomas Carey (University of Michigan, Ann Arbor, MI). Two additional cell lines of hematopoietic origin

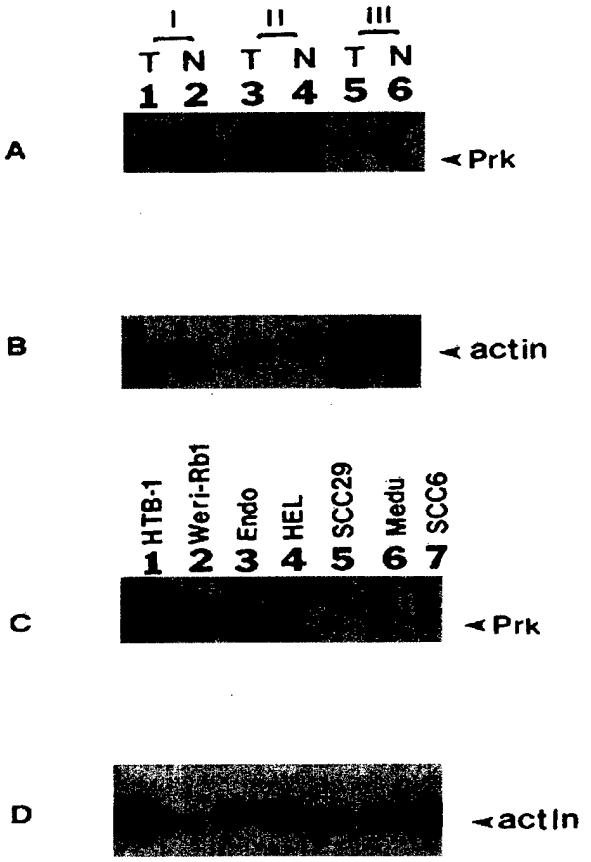

Figure 2. Northern blot analyses of *PRK* mRNA expression. Three examples of paired RNA samples from HNSCC patients were analyzed for *PRK* (**A**) or β-actin (**B**) expression. T stands for tumor-derived RNA and N for uninvolved normal tissues from the same patient. RNAs from various tumor-derived cell lines were analyzed for *PRK* (**C**) and β-actin (**D**) expression. UM-SCC29 and UM-SCC6 are two cell lines established from HNSCC samples.

TABLE 1. *PRK* Gene Expression in Head and Neck Squamous-Cell Carcinoma Patients

	RNA expression			
Stage	Not detected	Unchanged	Increased	Decreased
Primary tumor				
I				2
II	2			7
II	2	1		8
IV	1	2		5
Recurrence			1	4
Total (35)	5 (14%)	3 (8.5%)	1 (2.8%)	26 (74%)

(HEL, erythroleukemia, and Dami, megakaryoblastic leukemia) were also used for analyses. Adherent cell lines were cultured to about 80% confluence before harvesting for RNA analysis. Suspension cell lines were seeded at 2×10^5/ml and collected for RNA analysis when the cells were in logarithmic

growth phase. All cell lines were cultured in media containing 10% fetal calf serum. Northern blotting showed that *PRK* expression varied greatly among the cell lines analyzed (Fig. 2C and D). Little *PRK* expression was detected in UM-SCC29 (Fig. 2C, lane 5), whereas there was a moderate level of *PRK* expression in UM-SCC6. In contrast, the levels of *PRK* expression were very high in cell line HEL (Fig. 2C, lane 4). Interestingly, abnormal *PRK* transcripts (~ 2.9 kb) were detected in cell line Weri-RB1 (Fig. 2C, lane 2).

To determine whether deregulated *PRK* expression affected cell proliferation, we transfected A549 cells with expression constructs that encode either the full length (pcDNA3-PRK) or the short form (kinase inactive, pcDNA3-PRK-St) of *PRK* or with the vector alone (pcDNA3, Clontech, Palo Alto, CA) using lipofectamine (Gibco-BRL, Grand Island, NY) as a transfecting vehicle. The short form of PRK was obtained by removal of 23 N-terminal amino acid residues immediately after the methionine residue (Li et al., 1996). Stable transfectants were pooled, and an equal number of stably transfected cells were cultured in medium containing G418 (250 μg/ml). After 4 days of culture, A549 cells transfected with *PRK* cDNA grew much more slowly compared with those transfected with the vector alone or with the short form of *PRK* (Fig. 3A). Total RNA from each transfected population was also analyzed for *PRK* expression by Northern blotting. Because Dami cells express high levels of *PRK* transcript, DAMI cell RNA was used as a positive control. The near-full-length *PRK* cDNA (Fig. 3B, lane 4) was expressed as expected after transfection into A549 cells. Because the near-full-length *PRK* cDNA contains neither 5′ nontranslated sequence nor 3′ nontranslated sequence encompassing mRNA-destabilizing (ATTTA) motifs, the expressed *PRK* mRNA in transfected cells is shorter (Fig. 3B, lane 4) than its full-length cellular counterpart (lane 1). The proliferation experiment was repeated three times and similar results were obtained. In addition, inhibition of cell growth by constitutive *PRK* expression was also observed with GM00637D cells (data not shown).

The *PRK* gene encodes a protein of the polo kinase family (Li et al., 1996) whose members play a major role in regulating the G2/M checkpoint or mitotic progression (Fenton and Glover, 1993; Kitada et al., 1993; Kumagai and Dunphy, 1996). We have previously shown that the PRK protein level and its kinase activity are tightly regulated during the cell cycle (Ouyang et al., 1997). Many cell cycle regulatory genes (*RB1*, *p16*) or cell cycle

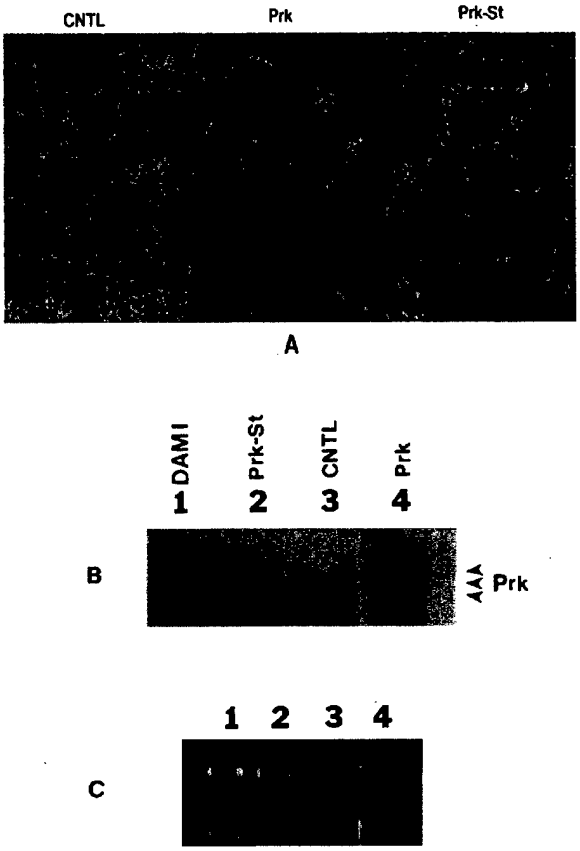

Figure 3. Inhibition of cell proliferation by constitutive expression of *PRK*. **A:** An equal number of A549 cells stably transfected with a full-length *PRK* cDNA (Prk), a truncated *PRK* cDNA (Prk-St), or the vector (CNTL) were seeded in culture plates. After 7 days' culture, overall growth patterns for each were recorded. **B:** Total RNAs from stably transfected A549 cells (lanes 2–4) were analyzed for *PRK* expression. Lane 1 denotes total RNA from megakaryoblastic leukemia Dami cells. **C:** Ethidium bromide staining of the RNA gel shown in B as a loading control.

checkpoint control genes (*TP53*, *ATM*) play a pivotal role in maintaining negative regulation of cell growth. Inactivation of these genes due to mutation, deletion, or their down-regulation often leads to malignant transformation (Sherr, 1996; Xu et al., 1997). We have reported that *PRK* expression is downregulated in a majority of lung carcinoma samples (Li et al., 1997). In the current study, we show that *PRK* is also downregulated in HNSCC, suggesting that compromised expression of *PRK* is correlated with the development of malignancy. Our FISH study reveals that the *PRK* gene localizes to 8p21, a chromosome locus that undergoes LOH in many types of human cancer, including HNSCC and lung carcinomas (Spurr et al., 1995; Bockmuhl et al., 1998), and that appears to harbor two putative tumor suppressor genes (Spurr et al., 1995; Imbert et al., 1996). Considering that constitutive expression of *PRK* significantly suppresses the prolifera-

tion of transformed lung fibroblast cells, it is tempting to speculate that the *PRK* gene may play an important role in regulating the rate of cell proliferation. Several genes, such as *p16* involved in negative regulation of cell proliferation, are significantly downregulated in many types of cancer (Sherr, 1996), and hypermethylation of the *p16* gene promoter is the primary mechanism that renders the gene transcriptionally inactive in tumors (Tasaka et al., 1998). Thus, it would be of considerable interest to examine whether hypermethylation plays a role in downregulating *PRK* expression in HNSCC as well as lung carcinoma patients.

The mechanism by which *PRK*, when overexpressed, exerts its antiproliferation effect remains unclear. It is interesting that *PLK* is capable of malignant transformation of mammalian cells when overexpressed (Smith et al., 1997). Constitutive expression of *PLK* in NIH3T3 cells causes formation of oncogenic foci that grow in soft agar and form tumors in nude mice (Smith et al., 1997). It is possible that *PRK* has a dual function by sensing the completion of genome duplication and initiating mitosis because its kinase activity peaks at late S and G2. If the *PRK* gene function is compromised and the above processes become uncoupled, the cell would undergo premature mitosis with an incompletely duplicated genome. This will lead to genome instability and predispose the cell to oncogenic transformation.

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